THE REVEALING OF CRYPTICITY OF PLASMID DNA HARBORED BY TWO STRAINS OF STREPTOCOCCUS FAECIUM

Inaugural-Dissertation
zur
Erlangung der Doktorwürde
der
Hohen Mathem. -Naturw. Fakultät
der
Rheinischen Friedrich-Wilhelms-Universität
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vorgelegt von

JORAM KENESS
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"... in pursuit of those far mysteries we dream of, or in tormented chase of that demon phantom that, some time or other, swims before all human hearts; while chasing such over this round globe, they either lead us on in barren mazes or midway leave us whelmed."

Herman Melville (1851)
"Moby Dick"

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PREFACE

The occurance of extrachromosomal DNA in bacteria has been shown to have wide reaching implications related to a variety of important phenotypes. These are: multiple antibiotic and metal ion resistance, production of toxins, antigenes, antibiotics, bacteriocins, conjugal donor ability and a diversity of metabolic capabilities.

Plasmid demonstration in any bacterial species can no longer be considered as an exceptional finding. Laufs and Kleimann (1978) report that among 186 selected gram negative and gram positive bacteria as many as 63% were found to harbor plasmids, 42% of the strains with extrachromosomal DNA showed more than one plasmid band in the agarose gel electrophoresis. The highest plasmid frequency was found in the eneteric, gram negative bacteria. 70-95% of the analyzed Enterobacter species, Klebsiella pneumoniae and Escherichia coli isolates harbored plasmids. The plasmid frequency in the Staphylococci and group D Streptococci ranged between 36-75%. Marder and Kayser reported 1977 the existance of large numbers of plasmids in Enterococci. Many of them carry resistance to antibiotics and some have been shown to transfer between enterococcal strains.

Clewell et al (1974), Corb and Murray (1976), Courvalin et al. (1972), Dunny et al. (1975), Jacob and Hobbs (1974), Jacob et al. (1975), Le-Blanc et al. (1978), Marder and Kayser (1977) and Van Embden et al. (1977) are the major contributors to plasmid studies in group D Streptococci.

The most frequent observation made within this group is: 1. plasmid determined antibiotic resistance, 2. plasmid coded bacteriocin production going hand in hand with hemolysin synthesis and 3. plasmid mediated conjugal transfer.

Due to the independent reproducibility of bacteriocin- and hemolysin cotransfer it is postulated by all above authors that both characteristics constitute two aspects of one and the same substance. It should be noted in this connection that the research teams citated above brought evidence for plasmid determined functions only for the genus S. faecalis and its varieties zymogenes and liquefaciens.

The object of this thesis was to reveal crypticity of "mini" plasmids harbored by S. faecium strains. These strains were isolated from patient's material and selected for their ability to synthesize bacteriocin by Brandis and Brandis (1963). Previous investigations (Keness et al. 1976) enabled reselection for those who possessed extrachromosomal DNA.

Our principle mode of investigation was based on mating experiments aimed at plasmid transfer via conjugation into appropriate recipient strains. We proceeded in comparing the isogenic pairs (differing only in the presence or absence of the plasmid/s) with respect to their phenotypic traits.

The plasmids involved in this dissertation are of a MW smaller than 15 Mdal. According to the existing models, this cathegory of plasmids lacks sufficient genetic information to promote direct genetic transfer. (Dougan et al. stated 1978, that conjugal plasmids are in general 40-70 Mdal in MW).

Falkow (1975) suggests that the term "nonself-transmissible" is more fitting to describe this phenomenon, because "mini" plasmids are known to be mobilizable via selftransferable plasmids (see Staudenbauer, 1978; Tomich et al. 1979; Williams et al. 1977 and Van Embden and Cohen 1973 among the many others).

Until some years ago, conjugal processes were thought to be restricted to the gram negative bacteria. The notion that conjugal DNA transfer could occur also among gram positive bacteria was first postulated by Raycroft and Zimmerman (1964). This group brought evidence for intraspecific transfer of chloramphenicol resistance markers in S. faecalis var liquefaciens. First evidence for a conjugal transfer process resulting in plasmid DNA transfer was reported by Jacob and Hobbs (1974). This group described plasmid mediated transfer of antibiotic resistance between two strains of S. faecalis. This report was followed by other publications indicating that the differences in cell wall structure (i.e. gram staining) does not constitute a factor influencing conjugation per se.

Conjugation was shown to involve aggregation mechanisms of the bacterial cells. This is believed to be a response promoted by proteinacous appendages of the donor cell called sex pilli. The ge-

netic information required for sex pilus biosynthesis, DNA transfer and other transfer functions, is encoded on conjugative or self-transferable plasmids by a continuous segment of DNA.
In the past few years a number of gene products involved in controlling and executing the mating cycle of the classic conjugative plasmid, the F sex factor of E. coli, have been identified (Dougan et al. 1978). However, similar evidence for the gram positive bacteria is still lacking.
The mode of conjugation within this bacterial group was partially investigated by Dunny et al. (1978). This team reports, that recipient strains of S. faecalis secrete a soluble substance which is trypsin sensitive, and which induces donor cells, carrying conjugative plasmids, to become very adherent. This is a phenomenon presumed to facilitate the formation of cell mating aggregates. Since cell free filtrates of recipients also elicited an aggregation (or clumping) response when mixed with donors, this substance was referred to as clumping inducing agent (CIA). We are not informed of any other studies elaborating on the mode of conjugation of gram positive bacteria.
Compared to the long employed modes of genetic transfer such as transformation, transduction and transfection, conjugation has proved to be as efficient (concerning rate of marker transfer) and as easily reproducable.
In the framework of this thesis plasmid DNA enrichment procedures were introduced. This became necessary because greater DNA quantities were needed for electrophoretic investigations. The electron microscopic preparation as well was altered to enable operation at lower magnifications. We proceeded in determining the sedimentation coefficient of the plasmids investigated in sucrose gradients. Subsequently the plasmid coded biological functions were revealed. This was performed in employing different conjugation- and reselection systems for plasmid DNA recipients. The DNA of the transconjugants formed was electrophoretically analysed and its origin, i.e. homology, was confirmed with the aid of the restriction endonuclease Eco RI.

MATERIAL AND METHODS

1. Bacterial strains

The strains investigated in this thesis were selected from the culture collection of the Institute For Medical Microbiology and Immunology of The University of Bonn.

S. faecalis strain JH2, originally characterized by J. Hobbs (1975), and the plasmids pAD1tc and pAMα1 (harbored by S. faecalis DT11) were a gift from Prof. D.B. Clewell of The University of Michigan.

The Enterococci were examined by Esper (1970) according to the methods described by Heeschen et al., 1967 (Which of their part, constitute a modification of the scheme published 1935 by Lancefield) and were typed as Streptococci of the serological group D (Antisera, Behringwerke). The biochemical differentiation resulted in their classification as Streptococcus faecium.

Streptococcus faecium 3-0 and S. faecium 25-0 were employed as bacteriocin- and plasmid donors. Besides S. faecium M16, we employed S. faecalis JH2 as plasmid recipient in genetic experiments.

Strain	Phenoytpe*
S. faecium 3-0	wild strain, bac^+
S. faecium 3-1	rif^r, fus^r derivative
S. faecium 25-0	wild strain, bac^+, suc^+
S. faecium 25-1	rif^r, fus^r derivative
S. faecium 158	wild strain, bac indicator
S. faecium 158-1	ery^r derivative
S. faecalis JH2	wild strain
S. faecalis JH2-2	rif^r, fus^r derivative
S. faecalis JH2-3	sm^r derivative
S. faecalis JH2-4	ery^r derivative
S. faecalis DT11**	ery^r
S. faecium M16	wild strain, ery^r, tc^r

*Abbreviations: bac^+ = bacteriocin producer; rif = rifampicine; fus = fusidic acid, suc^+ = sucrose utilizer; ery = erythromycin; tc = tetracycline, sm = streptomycin; x^r = resistant.

** new designation for the transconjugant S. faecalis JH2-2 harboring the erythromycin resistance determining pAMα1 plasmid.

The table seen in the previous page provides information with regard to the phenotypic traits which characterize the strains (and their derivatives) applied in this thesis.

2. Growth media

2.1 Routinely employed cultivation media

Complex medium

For the preservation of the streptococci in our strain collection we applied Tryptose Phosphate Broth (TPB, Difco Laboratories). This medium consists of 2% tryptose, 0,2% glucose, 0,5% NaCl and 0,25% Na_2HPO_4 (pH 7,3). The medium was solidified by supplementing it with 1,4% agar (Difco) where required.

M9 medium

The medium was applied as basal medium for radioactive labeling of DNA and for cultivation of strains aimed at subsequent enrichment of their plasmid DNA. The following formula is a modification by Clewell (1973) of the original one described by Kornberg 1959: The following solutions were separately autoclaved:

1. 20% glucose
2. 0,1 M $MgSO_4$
3. 0,01 M $CaCl_2$
4. Salt solution (vol 500 ml):
   30 g. Na_2HPO_4; 15g. KH_2PO_4
   2,5 g. NaCl and 5 g. NH_4Cl
5. complex ingredients:
   (per 430 ml. aq. dest.)
   1,5 g. yeast extract
   5.0 g. Casamino acids

10 ml of solution Nr. 1, 5,0 ml of each 2 and 3 and 50 ml of solu tion 4 were pipetted into solution Nr. 5 which was now ready for use.

2.2 Blood Agar

950 ml of the basic medium contained 10 g meat extract (Lab Lemco), 10 g peptone (Oxoid L37), 5 g NaCl and 15 g Agar (Oxoid 1). The ingredients were dissolved at 100°C and autoclaved subsequently for 15 min at 121°C abd 1,15 atmospheres. The medium was than cooled down to 50°C after which, according to our purposes, 50 ml defibrinated horse- or sheep blood was added to it.

2.3 Basal media for carbohydrate utilization studies

Cystin Trypticase Agar (BBL)

For the purpose of screening sucrose utilizing strains we employed CTA as basal medium. 900 ml of this semi-solid medium contained 0,5 g cystin, 20 g trypticase peptone, 5 g NaCl, 0,5 g Na_2SO_3, 17 mg phenol red and 2,5 g agar.
After dissolving the substances at 100°C and subsequent autoclaving, it was supplemented with 100 ml of a 20% sucrose solution in H_2O which was streily filtered beforehand.

Peptone Water (Oxoid)

Peptone Water was employed as basal medium for quantitative testing of sucrose utilization. Its formula per 1000 ml:
10 g Peptone (Oxoid L37), 5 g NaCl and 6,85 g (equivalent to 0,02 M) sucrose. Sterilization was carried out after thoroughly mixing at 121°C/1,15 atmospheres and 15 min.

Nutrient Broth

Nutrient Broth was employed as basal medium for all other carbohydrate utilization tests. 1000 ml of this medium consisted of 1,0% of the carbohydrate to be tested, 25 g Nutrient Broth (oxoid) and 32 mg brome cresol purple.

2.4 Ingredients added to the media - antibiotics:

For the purpose of resistance marker's selection as well as a selection pressure for plasmid DNA that codes for antibiotic resistance, the following antibiotics (combined or each at a time) were added to the medium employed: tetracycline (10 µg/ml), erythromycin (10 µg/ml), streptomycin (25 µg/ml), rifampicin (20 µg/ml) and fusidic acid (20 µg/ml). The substances were weighed for 500 ml medium, dissolved in 4,0 ml methanol and poured into the broth or agar which were kept at 45°C. At this temperature the methanol could easily evaporate.

3. Measuring bacterial growth:

The turbidity of the cell suspension was read at 530 nm in a Bausch & Lomb photometer (Spectronic 20) in tubes which had a

diameter of 15 mm. Measurements carried out in the Zeiss Spektral-Photometer (PM Q) applied glass cuvettes which were 1,0 cm thick. The wave length was the same.

4. Determination of minimal inhibition concentration of antibiotics:

Determination of MIC of antibiotics was carried out in TPB. 0,05 ml of a 1:100 diluted o.n. culture of the strain to be tested were pipetted into 5,0 ml of each of the antibiotic concentrations and subsequently incubated at 37°C. Evaluation was carried out at the moment the culture in the control tube reached exponential phase of growth. (ΔE_{550nm}= app. 0,7-0,85).
Water insoluble antibiotics such as rifampicine, tetracycline and fusidic acid were dissolved in methanol. The antibiotic stock solution was prewarmed to 42°C after which the alcohol easily evaporated and we could proceed with the dilutions.

5. DNA isolation techniques

5.1 The density gradient method of isolating purified plasmid DNA

Preparation of plasmid DNA involved the inoculation of 0,01 ml of an o.n. culture into 15 ml of medium supplemented with 7,5 uCi/ml of ^{3}H-thymidine. The cells were allowed to grow for about 6 hrs. (ΔE_{550nm}= 0,75), than centrifuged and washed with TES-buffer (0,05 M Tris-base, 0,05 m NaCl, 0,005 M Na_2EDTA pH 8,0). The cell pellet resuspended in 0,25 ml TES containing 200 mg sucrose/ml, to which 0,25 ml TES supplemented with 6 mg lysozyme/ml and 200 mg sucrose/ml, were added. Spheropiast formation was carried out for 20 min under slight agitation in a water bath. We proceeded in adding 0,25 ml of a 0,25 M Na_2EDTA (pH 8,0) to the above solution and continued with the incubation for additional 20 min. At the end of this step the cells were lysed by the addition of 0,25 ml of a detergent mixture consisting of sarkosyl/PEG (2,5%: 2,5%). Cell disrupture was followed by the clearing of the lysate.

For shearing the DNA a 5,0 ml syringe with a Nr. 1 gauge was employed. The cleared lysates were forced through the needle for 4 to 6 times until viscosity was fully lost, and then mixed with the density gradient compounds consisting of: 7,65 g CsCl, 1,5 ml

ethidium bromide (stock solution 1 mg/ml TES-buffer) and 5,5 ml. TES-buffer.
Centrifugation was carried out in 12,0 ml polyallomer tubes which were overlayed with paraffin oil and placed in a fixed angle rotor of the Heraeus Christ Vacuofuge - ultracentrifuge. The run was performed at 100,000 g (36000 rpm) for 45-60 hrs. at 20°C.

After bringing the run to a halt the tube's bottom was pierced with an MSE tube piercer and fractions consisting of 6 drops were collected directly into microtiter plates.
For the purpose of measuring radioactivity in the density gradient fractions; 20 ul of each of them were spotted on Whatman chromatography filter Nr. 1 cut in squares (2 x 1 cm) and placed on tinfoil. After drying them at 60°C for 20 min the filters were washed in 10% TCA then in ethanol and subsequently in ether. Hereafter they were placed in plastic vials which were filled with 10 ml of scintillation liquid (Aquasol, NEN chemicals). Measurement of radioactivity was carried out in a Beckman Model 230 LS liquid scintillation counter for 10 min. or until a failure rate of ±5,0% was reached.

5.2 Preparation of large DNA quantities

0,7 ml of an M9 o.n. culture of the strain to be tested were inoculated into 75,0 ml of m9 medium. Depending on the plasmid coded antibiotic resistances, the appropriate antibiotics were added to the medium as selection pressure. In cases in which further purification of DNA with CsCl density gradient were desired, the medium was supplemented with tritiated thymidine (2,5 uCi/ml). Incubation of the culture was carried out in a water bath at 37°C under slight agitation until ΔE_{550nm} = 0,75 (5-6 hrs.). The cells were subsequently harvested, washed once in TES-buffer and recollected in 1,5 ml TES containing sucrose (200 mg/ml). The hypertonic solution was placed into a 25 ml flask to which 1,5 ml TES supplemented with lysozyme (6mg/ml) and sucrose (200 mg/ml) were added. After an incubation for 20 min. at 37°C, EDTA (1,5 ml of a 0,25 M solution, pH 8,0) was pipetted onto the spherolplast forming solution and reincubation followed for 20 more minutes. Spheroplast disrupture was induced by 1,5 ml of a detergent solu-

tion consisting of sarkosyl/PEG (2,5%:2,5% in H_2O) and the lysates cleared usually at once. In some cases additional shaking at 37°C for 10-15 min. improved the clearing process.
The cleared lysates were frozen immediately hereafter at -20°C until shearing and DNA isolation followed.

5.3 Plasmid DNA enrichment procedure

After shearing the cleared lysate with a gaugeless 20 ml syringe for 20-30 times, it was diluted with TES buffer to a total of 70 ml.
Alkaline denaturation was performed with 3 M NaOH until a pH of 12,3 ± 0,2 was reached. The suspension was stirred at this level for 10 min. and subsequently renaturated with 2 M of Tris-HCl to a pH of 8,3 ± 0,2. At the end of this step cristalline NaCl was added to a total of 3% and we proceeded with the phenol extraction.

The phenol (analytical grade) was first melted in its original flask at 43°C in a water bath. The dense and clear liquid was then saturated with prewarmed (43°C) TES-buffer containing 6% NaCl by intensive shaking of equal volumes. The phenol separated rather quickly to form a clear and colorless phase at the bottom of the flask. The flask itself was then coated with tinnfoil to avoid light oxidation and kept at room temperature. (Temperatures lying lower then 18°C caused the phenol to become turbid and hampered its separation capacity following the extraction procedure).
The alkaline denatured cell extract was mixed in a ratio of 2:1 with the so prepared phenol and shaked gently for 2-3 min. Subsequently the milky solution was divided into tubes and centrifuged at 2000 g for 20 min. (room temperature). The aquous solution formed at the upper part of the tube was then pooled and extracted with half the volume of chloroform/isoamylalcohol and centrifuged at the same g level for 5 min. The plasmid DNA containing upper phase was pooled again and mixed thoroughly with twice the volume of ethanol and 0,1 volume of 3 M CH_3COONH_4 (pH 6,8). This procedure was concluded with a 48 hrs storage at -20°C, after which plasmid DNA percipitates were pelleted in the ultracentrifuge (100,000 g, -5°C and 60 min). The supernatant was carefully poured away and the tube dried with filter paper to avoid

residues of ethanol which would hamper DNA solubility.
The pellet was resuspended in TES-buffer, usually in 150-200 µl to get 1 µg DNA/1 µl. Quantitative DNA determination was carried out in 150 µl cuvettes assuming 1 µg/ml at ΔE_{260nm} = 0,2. These measurements were carried out in a PMQ spectral photometer.
The plasmid DNA solution was immediately hereafter divided into 20 ul portions and stored in plastic tubes at -20°C until used.

6. Sucrose gradient analysis of plasmid DNA

Sucrose gradient centrifugation was performed in a Heraeus Christ Vacuofuge ultracentrifuge using a swing-out-bucket rotor at 40000 rpm (=160000g), 20°C for 50 (alkaline-) to 60 minutes (neutral sucrose gradients).
A linear 5%-20% sucrose gradient in TES-buffer was employed to determine the sedimentation coefficient of the DNA under investigation. Plasmid pAM 1 isolated from S. faecalis DT11 was used as marker and labeled therefore with ^{14}C-thymidine.
Alkaline sucrose gradients consisting of 1,0 M NaCl, 0,3 M NaOH, 0,001 Na_2EDTA (pH 12,0) and 5%-20% sucrose, were employed to enable subsequent differentiation between the plasmid peaks (CCC and OC forms), as were observed in the neutral sucrose gradients.

100 ul of the plasmid DNA isolated with the CsCl-EB-density gradient method were mixed with 100 ul of the reference plasmid and overlayed on top of the gradient. After centrifugatin as indicated above, the bottom of the tube was punctured and fractions (consisting of one or two drops) were collected directly on Whatman chromatography filter Nr. 1 cut into squares.
The filters were subsequently dried and washed in TCE, ethanol and ether (as indicated under 5.1). The gradient itself (vol. 4,8 ml), was formed in a MSE gradient former apparatus and the linear uptake of sucrose concentration was tested with an Abbe refractometer (Zeiss, Oberkochen) at 20°C.

7. DNA preparation for electron microscopy

5-50 µl of DNA were mixed with 750 ul of a freshly prepared solution of cytochrome C (2 µg/ml 0,15 M CH_3COONH_4 containing 0,5% formaldehyde). 50 ul drops of this solution were placed on the

surface of a teflon block (cleansed by treatment with chromsulfuric acid prior to use), and were kept under cover at room temperature for 20 min to allow surface formation of a monomolecular cytochrome film and simultaneous spreading of the attached DNA strands.

By contacting a formvar-carbon-coated grid with the surface of one drop and gently withdrawing, the protein monolayer with the DNA spread on it was transferred onto the grid.

Precontrastation of the DNA was performed by staining the specimens in uranyle acetate (0,002% in ethanol) for 30 sec. and subsequent rinsing in isoamyl alcohol for 10 sec. Residula fluid was removed by a tip of a filter paper.

Conical shadowing with platin-palladium:

The coating material, 6 cm wire (0,2 mm Ø) of platin-palladium alloy (1:4), was tightly wound around a tungsten rod (0,5 cm Ø), the latter serving as an evaporation source in a Blazers vacuum coating unit. The specimens were fixed on a turntable at a distance of 10 cm from the evaporation source. Evaporation was operated at a vacuum of 10^{-5} atmospheres. The turntable bearing the specimen tilted from its vertical position at an angle of $7\text{-}10^{\circ}$ towards the evaporation source, rotating at a 110 rpm. The process of conical shadowing was completed within 3 minutes.

The shadowed DNA specimens were examined in a Siemens Elmiskope 101 at a high tension of 80 kV and an aperture of 50 µ. Pictures were shot at magnifications varying between 25,000-50,000 times.

Concerning plasmid DNA length determination, the marker plasmid was mixed in a relation of 1:1 with the DNA of the unknown plasmid before proceeding with the shadowing.

8. Selection mode of antibiotic resistant mutants

Rifampicin resistant mutants were selected on TPB agar containing 25 µg/ml of the substance. A 5,0 ml o.n. culture was spun down, (in a Heraeus Christ Sputnik centrifuge), the pellet recollected in 1,0 ml of sterile broth and 0,1 ml portions were pipetted on the surface of the selective agar. Evaluations of mutants was carried out 48 hrs. later to allow phenotypic expression.

Mutants resistant to erythromycin or fusidic acid had to be selected from 50-100 ml cultures (the cells of which had to be resus-

pended in 1,0 ml following centrifugation) because of the very small rate of spontaneous resistance towards these substances.

9. Bacterial mating procedure

Bacterial mating was performed principally on filters. 0,25 ml of an o.n. culture (5,0 ml, M9 medium) of both donor and recipient strains were mixed with 4,5 ml of the sterile broth and filtered through a 25 mm diameter, 0,45 μ pore-size membrane filter. (Millipore, HAWP 01300, Millipore Swinnex/13 filter unit).
The filters were subsequently carefully placed on agar plates and incubated for 18 hrs. at 37°C. Mating mixtures were removed from membrane filters by vigorously mixing it (Vortex Genie) in 1,0 ml of the basal medium. Cell suspension were now serially diluted and 0,1 ml samples were plated on the solid medium containing the selective agents for the transconjugant's selection.
Previous to mating, it was determined whether or not donor or recipient strain produced bacteriocin towards one another. If so, donor and recipient were mixed in a relation of 1:10 (0,05 ml + 0,45 ml).
Each experiment included sets of negative as well as positive controls. Due to the fact that the transconjugant's selecting agents consisted mainly of antibiotics, it was of great importance to follow spontaneous mutation to resistance. In addition, both donor and recipient strains were incubated separately in the same fashion as the mating mixture.
Selection of rifampicin- and fusidic acid resistant S. faecium 3-1 transconjugants harboring pAD1tc plasmid were carried out on agar containing tetracycline (10 μg/ml), rifampicin and fusidic acid (each 25 μg/ml). The same selecting agents were involved in selecting S. faecium 25-1 transconjugants harboring the pAD1tc plasmid. The transfer of pAD1tc from S. faecium 3-1 or 25-1 transconjugants to S. faecalis JH2-3 was selected for on tetracycline and streptomycin containing agar.
Transconjugants of S. faecalis JH2-2 (rifampicin- and fusidic acid resistant) apparently containig pM16 plasmid were screened for on agar supplemented with the latter two antibiotics as well as either erythromycin or tetracycline or both these antibiotics together.

10. Plasmid DNA curing procedure

For the purpose of curing antibiotic resistance, which was apparently plasmid bound, ethidium bromide was employed as mutagenic agent. 0,1 ml of an o.n. culture of the strain concerned were inoculated into 5,0 ml of TPB supplemented with EB (3,6 µg/ml) and grown till a late exponential phase. After about 18 hrs. the culture was diluted to about $3 \cdot 10^2$ CFU/ml and 0,1 ml portions were plated on TPB agar (=master plates). Incubation followed o.n. after which replica plating on antibiotic supplemented agar was performed. Both master- and daughter plates were reincubated o.n. and on the following morning the CFU, which grew only on the master plate, (and thus lost their antibiotic resistance markers), could be made up.

11. Bacteriocin characterization

11.1 Bacteriocin test assay

For the purpose of testing bacteriocin production of CFU a TPB-agar plastic dish was divided clockwise into a maximum of 8 fields and the CFU were inoculated with a needle at the center of every such field. After o.n. incubation at 37°C the plates were turned face down on a metall grid (capable of carrying 5 petri dishes at a time) that was placed on top of a container filled up with chloroform to 1,0 cm from its edge. The container was placed in a ventilated hood. After 45 min. the plates were removed and subsequently overlayed with 2,5 ml of soft agar (0,7%) containing 0,1 ml of an o.n. culture of the indicator strain. This step was followed by a two hour diffusion of bacteriocin at 4°C. An o.n. incubation at 37°C, after which inhibition zones were visible, proceeded.

11.2 pH stability

A 70,0 ml culture of the bacteriocin donor was allowed to grow till a late logarithmic phase was reached ($\Delta E_{550\ nm} = 0,9$). The cells were then spun down and the supernatant heated for 10 min at 80°C. Subsequently the supernatant was divided into 5,0 ml portions and their pH adjusted with either 0,1 N HCl or 0,1 N NaOH to the following levels: 1,2,3,4,5,6,7,8,9,10,11 and 12. Each one of them was serially diluted (1:2, 1:4...1:128) abd 10 ul of every dilution step spotted on TPB plates which were overlayed with 0,1 ml.

of S. faecium 158 (indicator strain) o.n. culture in 3,0 ml soft agar. Negative controls consisting of 0,1 N HCl and 0,1 N NaOH (10 µl of each) were carried out as well. The petri dishes were placed for two hrs. at 4°C to allow bacteriocin diffusion. This was followed by subsequent incubation at 37°C for 18 hrs.

11.3 Sensitivity towards proteolytic enzymes

3,0 mg of the following enzymes were dissolved in 1,0 ml of the bacteriocin containing supernatant (which had been treated for 10 min at 80°C beforehand): pepsin, trypsin, chemotrypsinogene and pronase. This step was followed by an incubation at 37°C for 30 min. The enzymatic degradation was brought to a halt by abrupt freezing at -20°C (in dipping the tube in cold ethanol). A serial dilution followed (see pH stability) and negative controls consisting of: A. each of the enzymes dissolved in sterile TPB in the above mentioned concentration; prefrozen (-20°C) B. enzymatically not digested but prefrozen bacteriocin, were performed. The sensitivity of S. faecium 158 towards the so treated bacteriocin was tested as mentioned above.

12. DNA electrophoresis procedure

Ethanol percipitated DNA (15-30 µl) was electrophoresed in 1,0% agarose (Saakam, Marine Colloids) dissolved in 89 mM Tris-base, 2,5 mM Na_2EDTA and 8,9 mM boric acid. The DNA samples were mixed with a quarter of their volume in a solution containing brome-cresole purple (0,07%), serving as migration fron marker, sodium dodecyle sulfate (7%), glycerol (33%) and distilled water. Electrophoresis was carried out in a vertical slab gel (Bio-Rad Laboratories, Model 220). The dimensions of the gel were 14x12x0,3 cm. Sample wells were formed by 8 polypropylene-made sticks each 0,9 cm wide. Power supply was furnished by a Bio-Rad model 400 apparatus and electrophoresis was carried out at 17°C as followes: 120 V and 35 mA for 15 min (until the dye penetrated the gel), after which the electrophoresis was adjusted to 20 V 4 mA for 22 hours.

DNA specimen which were investigated for the first time and samples containing plasmids with a known MW greater then 25 Mdal were first electrophoresed for 1 hr. at 120 V/35 mA and subsequently

for 20 hrs. at 20 V/4 mA (making 1,25 V per well).
After completion of run the gel was placed in a solution containing 1 µg/ml EB to stain the DNA. (2-24 hrs. depending on DNA concentration).

13. DNA cleavage procedure with Eco RI

2,0 µl of Eco RI (Boehringer Mannheim; $3\text{-}9 \cdot 10^4$ U/ml) were mixed with 3,0 µl of the reaction mixture consisting of 0,1 M Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$ pH 7,5 and 6,0 µl of the dilution buffer (10 mM KH_2PO_4, 0,2 M NaCl, 1 mM EDTA, 0,7 mM mercapto ethanol, 2 mg/ml bovine serum albumin and 0,2% triton, pH 7,0)
20-30 µl plasmid DNA (= aaprox. 20 µg DNA) were thawed and placed in a microtiter plate. The Eco RI solution was then added and a 45 min incubation under slight agitation followed at 37°C. Remixing was undertaken every 15 min and the reaction was brought to a halt with 10 ul of a solution consisting of 7 M Urea, 50% sucrose, 0,1 M EDTA pH 7,0 and 0,1% brome phenol blue.
Electrophoresis of the so digested plasmid DNA followed subsequently. At the same time an Eco RI digest of Lambda-phage DNA was run in a parallel slot as length (and hitherto MW) marker.
The preparation of Lambda-phage-DNA length standard consisted in dissolving the lyophilisate (Boehringer Mannheim) in 50 µl redistilled water that included 0,15 M NaCl and 0,015 M sodium citrate. 1,0 µl herefrom (Hamilton syringe) were mixed with 10 µl of the Eco RI stopper solution and applied on the gel slot.

14. Sucrose utilization assay

The strain to be tested was grown o.n. in peptone-water (Oxoid) supplemented with 0,02 M sucrose. 100 ml of this medium were inoculated with 1,0% and immediately a 5,0 ml sample was removed to determine sucrose concentration and optical density (550 nm, Bausch and Lomb Spectronic 20). Cultivation was carried out at 37°C under slight agitation till a late stationary phase of growth was reached. Approximately every 45 min (see results) 5,0 ml samples were taken, their optical density measured and subsequently centrifuged (5000 rpm/20 min). The supernatant was frozen at -20°C and kept this way until quantitative sucrose determination was performed.

14.1 Quantitative sucrose determination

The test is based on measuring glucose concentration before and after enzymatic hydrolysis via increase in optical density of NADPH (at 340 nm). In this system, the concentration of NADPH increases stoichiometrically to the amount of released glucose.
Test specifications:

	blank	glucose sample	sucrose sample
ß-Fructosidase: 72 U/ml 0,32 M citrate buffer pH 4,6	0,2 ml	-	0,2 ml
Culture supernatant (1:40 diluted)	-	0,1 ml	0,1 ml

The solutions were mixed and allowed to stay for 15 min at 25°C after which the following reagents were added:

3 mM NADP, 12 mM ATP, 10 mM $MgSO_4 \cdot 7H_2O$ in 0,75 M triethanolamine pH 7,6	1,0 ml	1,0 ml	1,0 ml
Redistilled water	1,8 ml	1,8 ml	1,8 ml

3 min after mixing the O.D. was read. (ΔE1 at 340 nm, Zeiss Spectral Photometer PM 7). The reaction was started by the addition of:

HK 290 U/ml, G-6-P-DH 145 U/ml, both dissolved in phosphate buffer pH 7, 0,1 M.	0,02 ml	0,02 ml	0,02 ml

The test assays were carried out in glass cuvettes with a light pass 1,0 cm and a volume of 3,5 ml. Temperature was held constant at 30°C during O.D. reading by the PM 7 spectral-photometer.
After the enzymatic process terminated (20-30 min) ΔE2 was read and and ΔE for sucrose calculated as follows:

δE sucrose = δE_2 total glucose - δE_1 glucose

Absolute sucrose concentration was calculated with the aid of the following formula:

$$C_{g/l} = \frac{V \cdot MW \cdot F \cdot \delta E \text{ sucrose or } \delta E \text{ glucose}}{\varepsilon \cdot d \cdot v \cdot 1000}$$

whereas:

V = final volume (3,02 ml)
v = sample's volume (0,1 ml)
d = light pass (1,0 cm)
ε = extinction coefficient of NADPH at 340 nm (6,3 l $mmol^{-1} cm^{-1}$)
F = dilution factor (1:40)

MW = molecular weight of the substance assayed for. (sucrose: 342.3; glucose: 180.16)

15. Quantitative protein determination (Beisenherz et al, 1953)

The Biuret reagent consisted of 9,0 g K-Na-Tartrate, 3,0 g $CuSO_4 \cdot 5H_2O$ and 5,0 g KJ.

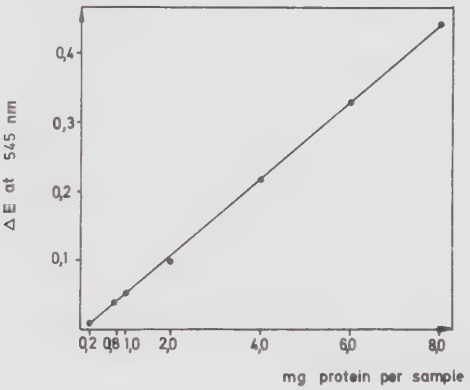

Figure 1: Protein reference curve prepared employing bovine serum albumin.

Protein samples were pipetted into centrifuge tubes filled with distilled water to a total of 1,0 ml and mixed thoroughly with 0,15 M TCA (3 M). The precipitated protein was centrifuged at 5000 rpm for 20 min. and the pellet resuspended in 2,0 ml H_2O

to which 3,0 ml of the Biuret reagent were added. After 30 min of incubation at room temperature the extinction of the samples was read at 545 nm against a blank sample containing Biuret reagent and water in a ratio of 3:2.

16. Preparation of crude cell extract for enzymatic investigations

Peptone-water broth supplemented with 0,02 M sucrose was inoculated (1%) with an o.n. culture of the strain to be tested.
After reaching growth phase, in which sucrose utilization had reached its maximum (As determined in appropriate experiments, see results), the cells were harvested, washed twice in phosphate buffer (0,1 M pH 7,0) and their wet weight determined. Each gram of cell wet weight was recollected in 3,0 ml of cold buffer and subsequently sonified with a Branson sonifier at maximum power output and 15 min (with 1 second intervals). The temperature during sonification was kept constant at 2°C with the aid of an Ultrakryomat TK 30D having ethanol as cooling agent. Quality of disruption was checked microscopically in 20 min. intervals.
Cell disruption with the aid of a French Press (Cell Press, American Instrument Company, Silver Spring) proved unsuccessful. A three time passage through the press at 1400 atmospheres resulted in the breakage of less then 10% of the cells, probably due to their round form.
Following ultra sonic treatment the suspension was centrifuged at 30,000 g for 15 min (Sorvall RC-2B) to pellet whole cells and cell debris. After determining protein concentration (Biuret method) the supernatant was portioned to 0,1 ml samples which were transferred into small plastic tubes and frozen at -20°C until the enzyme assaywas performed.

17. ß-Fructosidase assay (Bergmeier and Berndt 1974)

Prior to the determination of ß-Fructosidase activity the presence of glucose in the supernatant of the crude cell extract had to be investigated. For this purpose 250 µl of the extract were boiled at 100°C fo5 5 min to abolish any enzymatic activity. The glucose test was carried out exactly as described in the ß-Fructosidase assay (see next page) with the following exceptions:

1. 0,1 ml of the supernatant (crude cell extract) pretreated as mentioned before, were pipetted into the blank as well as into the glucose sample and 2. the addition of sucrose was omitted.

The reaction was started with G-6-P-DH + HK and the ΔE was read at 340 nm 30 min later in the Zeiss spectral photometer PM 7.

The evidence of the presence of ß-Fructosidase in the supernatant was arrived at by means of the following test scheme:

	conc. in the test	blank	suc, sample
supernatant of crude cell extract	app. 250 µg	10-25 µl	10-25 µl
2 mM sucrose	63 uM	-	0,1 ml
0,1 M citrate buffer pH 4,6	32 mM	0,3 ml	0,3 ml

The solutions were mixed and allowed to stay for 15 min at 25°C after which the following reagents were added:

0,75 M triethanol amin pH 7,6 10 mM $MgSO_4 \cdot 7H_2O$	0,24 M 3,2 mM	1,0 ml	1,0 ml
redistilled water	-	1,6 ml	1,5 ml
12 mM NADP	0,38mM	0,1 ml	0,1 ml
81 mM ATP 0,6 M $NaHCO_3$	26 mM 0,19 M	0,1 ml	0,1 ml

O.D. was read at 340 nm three min. after mixing the samples. The reaction was started by adding the following:

2 mg/ml HK 1 mg/ml G-6-P-DH	$1,8 \cdot 10^3$ U $0,9 \cdot 10^3$ U	0,02ml	0,02ml

Optical density (ΔE_2) was read at 3 seconds intervals. The enzymatic reaction was carried out in a constant temperature of 25°C. This was achieved with the aid of a thermostate connected to the cuvette.

NADPH concentration was calculated as follows:

$$\frac{(\Delta E_2 - \Delta E_1 - \Delta E_o) \cdot 3{,}14}{6.22} = \mu M \text{ NADPH/sample}$$

18. Source of chemicals

Defibrinated horse blood and Peptone-Water from Oxoid/Wesel
Rifampicin was a gift from Chemie Grünenthal, Cologne.
Fusidic acid, erythromycin were purchased from Bayer Pharma
Tetracycline, ethidium bromide, Tris-HCl, EDTA, pepsin, trypsin, chemotrypsinogene from Serva/Heidelberg.
Restriction endonuclease, ß-Fructosidase, NADP, ATP, G-6-P-DH, HK and bovine serum albumin from Boehringer/Mannheim.
Lysozyme, pronase, CsCl and Na-Sarkosinate from Merck/Darmstadt.
^{3}H-thymidine, ^{14}C-thymidine from Amersham Buchler/Braunschweig.
TPB, yeast extract, casamino acids, CTA-medium and agar from Difco Laboratories/Stuttgart
Unisolve I scintillation cocktail from Zinsser/Frankfurt.
Agarose for electrophoresis from Seakam, Marine Colloids/Maryland.

EXPERIMENTS AND RESULTS

1. DNA alkaline denaturation and its effect on plasmid enrichment

In proceeding previous investigations concerned with plasmid DNA isolation from bacteriocinogenic S. faecium strains (Keness et al. 1978), it became necessary to modify the method described by Bazaral et al. (1968), aimed at optimizing plasmid yeald.

The relatively small amount of plasmid DNA isolated in the CsCl-EB- density gradient (100 µl with less then 2 µg DNA/ml/gradient) were sufficient for electron microscopic investigations but not for analytical electrophoresis. In as far as DNA isolation for electrophoretic investigation purposes was concerned, a compromise could be made: a higher DNA yeald could be arrived at at the cost of lower nucleic acid purity, i.e., density gradient centrifugation was not imperative any more and radioactive labelling could therefore be omitted.

S. faecium 25-0 was grown for the purpose of demonstration of DNA isolation in:
15 ml M9 medium supplemented with 7,5 µCi/ml ^{3}H-thymidine and in 50 ml M9 medium supplemented with 2,5 µCi/ml ^{3}H-thymidine.
Both cultures were inoculated with 0,5% of the 1:100 dilution of an o.n. culture and incubated under slight agitation at 37°C for 6 hrs. At this time optical density reached 0,70 (ΔE at 550nm) and the cells were harvested. If cultivation continued to O.D. exceeding 0,75, it became difficult to clear up the lysate through treatment with a detergent. This phenomenon was observed in particular while lysing S. faecalis strain JH2 and its derivatives. Cleared lysates from all S. faecium strains could still be ob - tained at an O.D. of 0,75 - 0,80 but cultures were rarely cultivated as far as this stadium.
The cell pellet was washed once with TES-buffer after which spheroplast formation with the aid of lysozyme in hypertonic sucrose solution and EDTA followed. Lysis was carried out with a detergent mixture (sarkosyl/PEC) and the cleared lysates sheared with the help of a syringe.
The crude DNA preparation from the first culture was immediately mixed with the CsCl-EB-density gradient solution while the lysate

from the 50 ml culture (consisting of 6 ml) had to be further purified before undergoing the same treatment. Purification meant extraction of a substantial amount of the superfluous chromosomal DNA besides the deproteinization.
Chromosomal DNA had already been cut into linear double strands by the intensive shearing. This step was proceeded by the alkaline denaturation (3 M NaOH, pH 12.3 for 10 min) which converted the double strands to single strands. Renaturation (Tris-HCl, pH 8,3) was followed by protein denaturation employing half the volume of TES-buffer and 3% NaCl saturated phenol. After intensive shaking (1 min) the phases were separated by centrifugation and the aquous supernatant further purified by another shaking with chloroform/isoamyl alcohol (24:1). Chloroform is used as a surface denaturant and to remove the denatured protein and phenol residuals, the alcohol is present to prevent frothing and to enable the the resulting emulsion to separate more readily.
The DNA containing upper phase was mixed thoroughly with twice the volume of ethanol (-20°C)/0,1 Vol CH_3COONa (pH 6,8) and allowed to precipitate for 48 hrs.
DNA pelleting was performed at 100,000 g/-5°C/60 min. The percipitate was subsequently dissolved in 1,0 ml TES-buffer and mixed with the CsCl-EB-density gradient compounds. Ultracentrifugation was carried out for 60 hrs. For evaluation of the radioactive marked fractions see material and methods.

Figure 2 presents the plotting of the radioactivity of the fractions won from the density gradient. Extrachromosomal DNA from the cleared cell extract (Fig 2 A) makes only 1/7 of the total nucleic acid while after enrichment, as presented in Fig 2 B, the amount of plasmid DNA reaches 2/3 of the total.
Radioactivity of the plasmid DNA fractions derived from the cleared cell extract shows 150,000 cpm at its peak. The parallel peak from the enriched preparation however, points at 850,000 cpm. This means a 5,6 fold increase in radio isotope concentration and hitherto hints at an increased amount of DNA. These findings are due among others to the 3,3 times greater culture volume of the enriched preparation, pointing out at the same time the efficiency of the purification.

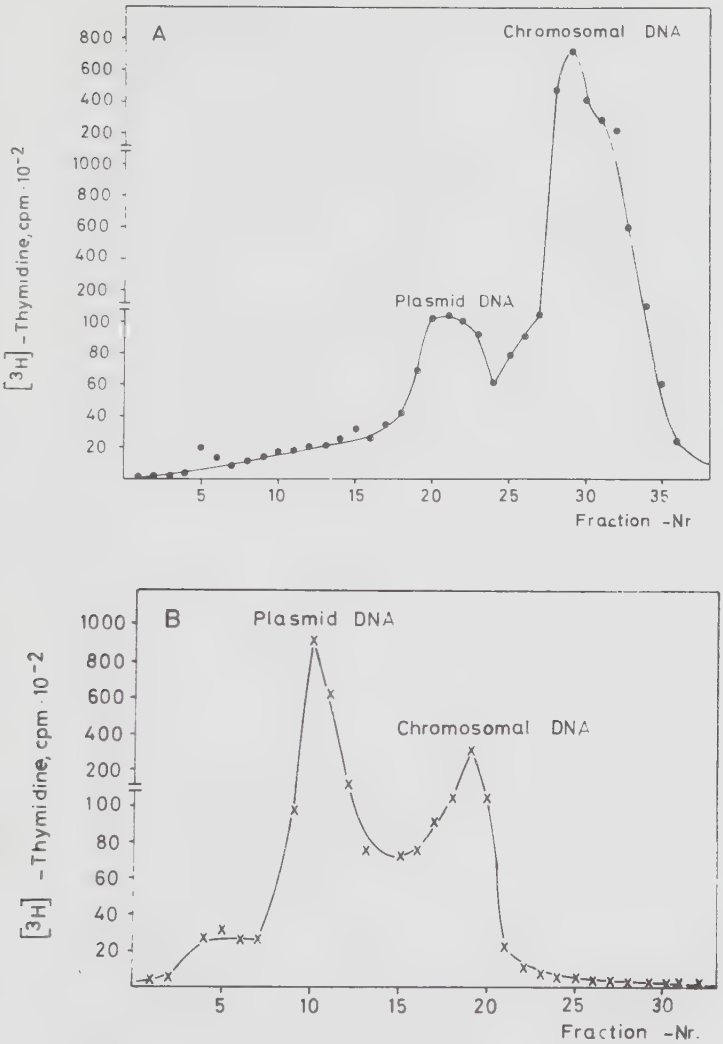

Figure 2: Direct cesium chloride - ethidium bromide density gradient centrifugation (A) and sedimentation profile following plasmid DNA enrichment through alkaline denaturation and phenol extraction (B)!
(For details see under material and methods)

2. Characterization of the biophysical plasmid properties

2.1 Electron microscopic investigation of plasmid DNA

For the purpose of electron microscopic DNA preparation, the plasmid DNA had to be free of chromosomal DNA. This was achieved by means of CsCl-EB-density gradient centrifugation.
S. faecium strain 3-0 and strain 25-0 as well as S. faecalis JH2-3pAD1tc were, for this purpose, cultivated in 70 ml M9 medium supplemented with 1 mCi/ml ^{3}H-thymidine. Incubation followed until ΔE_{550nm} = 0.75. After harvesting the cells in hypertonic sucrose-TES-buffer solution we lysed them employing lysozyme (6 mg/ml), EDTA (0,25 M pH 8,0) and sarkosyl/PEG.
shearing of the cleared cell lysate was followed by plasmid enrichment via alkaline denaturation which affected primarily chromosomal DNA. Cell debris and other proteins were extracted subsequently with phenol and phenol residuals with chloroform/isoamylalcohol. The DNA was percipitated with ice cold ethanol and the percipitates recollected in 1,0 ml of TES-buffer to be subsequently mixed with the CsCl-EB-gradient solution. Centrifugation followed at 100,000 g for 55 hrs and 18°C. After the evaluation of radioactivity of the gradient's fractions those containing plasmid DNA were pooled and dialysed against SCC buffer (0,015 M NaCl, 0,015 M Na-citrate) until no dye was visible (which took usually 4-5 hrs.)
For electron microscopic preparation we mixed 25 µl DNA of each of the S. faecium strains with 25 µl of the plasmid DNA isolated from S. faecalis JH2-3pAD1tc (employed as MW marker) and 750 µl cytochrom e. Preparation procedure followed the scheme presented in "material and methods".
Shadowing of the specimen with a platin-palladium-alloy was performed at a distance of 10 cm to the evaporation source (In prior shadowings the distance was kept at 16-18 cm). Plasmid screening was carried out at magnifications reaching 80,000 fold because of the fade contrast; in getting closer to the evaporation source the shadowing became more intense and the plasmids could be screened at magnifications ranging between 25-50,000 fold without effecting the quality of the picture.
The length of the plasmid DNA was measured with the a d of a map

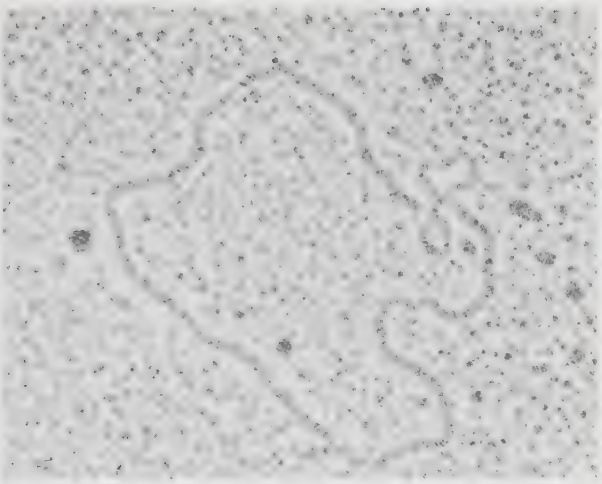

Figure 3: The open circular form of plasmid pJK3 isolated from S. faecium 3-0. Shadowing of the DNA was performed with a platin palladium alloy (x 160,000)

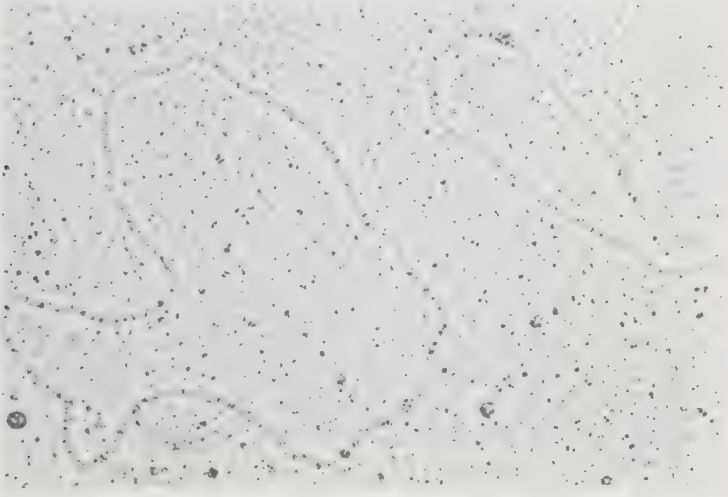

Figure 4: The "midi" and "mini" plasmids harbored by S. faecium strain 25-0. To the right a 2,3 μ plasmid (corresponding to a MW of 4,7 Mdal) and to the left the "mini"-plasmid with a length of 1,25 μ (corresponding to a MW of 2,4 Mdal. See text for calculation factor (x100,000)

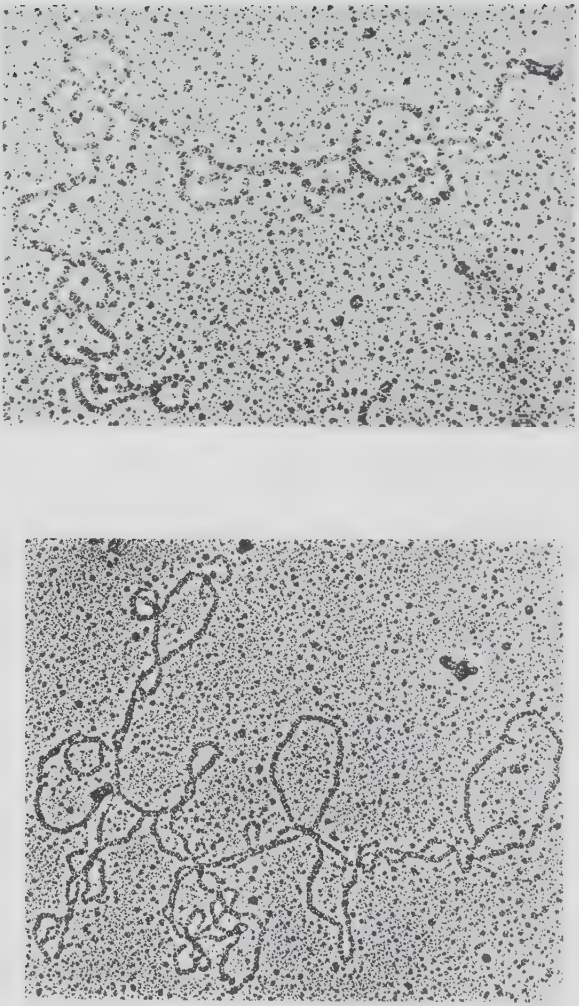

Figure 5: The "maxi" plasmid harbored by S. faecium 25-0 (MW 13,0 Mdal) in its covalently closed circular form (Top picture, x 100,000) and after beginning of relaxation (due to single strand nicks) as seen at the bottom picture (x 80,000)

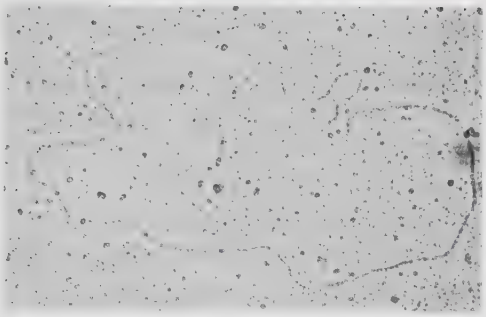

Figure 6: The maxi plasmid harbored by S. faecium 25-0 in its fully relaxed ("open-circular") form. (x 40,000)

distance measuring circle; plasmid pAD1tc with its length of 18,3 u was hereby employed as marker. It was chosen as such because according to electron microscopic findings it was the longest of all and could therefore be easily distinguishable as such. The plasmid size was determined in building an average of at least 10 measurements of plasmid copies. Calculation of molecular weight followed Lang (1970) whose findings showed that 1 μ of DNA is equivalent to a MW of $2{,}07 \cdot 10^6$ Dalton.

For S. faecium 3-0 the length of 1,7 $\pm$ 0,11 μ has been determined. This value is equivalent to a MW of 3,5 Mdal. S. faecium 25-0 mini plasmid was 1,25 $\pm$ 0,12 μ long which corresponds to a MW of 2,4 Mdal. The "midi" plasmid of the same strain is 2,3 $\pm$ 0,18 μ long which is equivalent to a MW of 4,7 Mdal; the "maxi" plasmid was found to be 6,3 $\pm$ 0,3 μ long corresponding to 13,0 Mdal. The relation of CCC to the OC plasmid form remained approximately the same.

2.2 Determination of sedimentation coefficients of plasmid DNA in sucrose gradients

In employing the sucrose zonal centrifugation an additional mode of plasmid DNA demonstration and differentiation of their tertiery structural forms was made use of. The forms concerned are super coiled or covalently closed circular (CCC) form, open circular (OC) due to single strand nicks of the CCC DNA and linear DNA. The more compact CCC form migrates quicker during centrifugation then the OC form; the latter creates rather flat peaks in the radiogramm according to Clewell et al. (1974). The linear DNA debris are found on the bottom of the tube under normal centrifugation conditions (Meaning 160,000 g/50 min). Under alkaline conditions (sucrose solution having a pH of 12,0) which lead to breakage of the hydrogen bonds between the two strands of a DNA duplex, the strands of the linear or OC duplexes are separated, but the strands of the CCC DNA remain interlocked. Thus, when centrifuged under alkaline conditions, each structure forms a compact random coil, the CCC form being a far more rapidly sedimenting circle then the OC form of the same plasmid (Clowes, 1972)
The sedimentation coefficient constitutes another important factor in the characterization of plasmids. The physical dimension is a function of the sedimentation velocity of bio-macromolecules and is expressed in Svedberg units (1 Svedberg = 10^{-13} sec).
The absolute value of the sedimentation coefficient can be determined by cosedimentation of a purified DNA standard of known S value. For this purpose pAMα1 isolated and characterized by Clewell et al. (1974) was employed. This plasmid codes for resistance towards erythromycin, has a MW of 6 Mdal and a sedimentation coefficient of 28 S for its CCC form.

2.2.1 Isolation of radioactively labelled plasmid DNA

For the purpose of isolating plasmid DNA the CsCl-EB-density gradient purification procedure had to be employed. We could perform the following investigations only with carefully isolated, pure plasmid DNA free of "chromosomal contaminants".
S. faecium 25-0 and S. faecium 3-0 were grown o.n. in M9 medium. 0,75 ml hereof were employed as inoculum for the main cultures.

(75 ml M9 medium). Both cultures were supplemented immediately after inoculation with ^{3}H-thymidine (2,5 µCi/ml). S. faecalis DT11 was grown in 15 ml M9 and supplemented with 10 ug/ml erythromycin (as selection pressure for pAMα1) and ^{14}C-thymidine (0,8 µCi/ml) in view of its function as sedimentation marker.
The cultures were incubated until $\Delta E_{550\ nm} = 0{,}75$. This was followed by harvesting and lysing the cells with lysozyme in hypertonic sucrose solution, EDTA and sarkosyl/PEG. (See material and methods for details). Following the DNA shearing the cleared lysate was mixed with the CsCl-EB-gradient compounds and centrifuged at 100,000 g for 45 hrs and 20°C. The tube's bottom was subsequently punctured and fractions collected in microtiter plates. 20 µl of each fraction were spotted on filter squares which were subsequently washed (TCA followed by ethanol and ether) dried, and placed in scintillation vials for measurement of radioactivity.

Figure 7 represents the sedimentation profile of the DNA isolated from S. faecalis DT11: the pAMα1 plasmid peak is clearly observed. The indicated fractions were pooled and dialysed against SSC-buffer for 4 hrs. as was done with all other plasmids isolated in

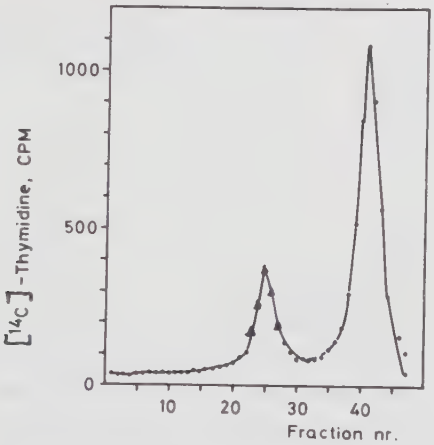

Figure 7: CsCl-EB-density-gradient centrifugation of the cleared cell lysate from S. faecalis DT11 (density uptake from left to right). The fractions marked "▲"were pooled and processed as indicated in the text.

this experiment. Within this time the EB had diffused away and the plasmid DNA could be applied on the sucrose gradient. Concerning the S. faecium cultures, their lysis had to be followed by alkaline denaturation because of their 5 times greater culture volume compared to S. faecalis DT11. Aside from this there was the very high density of the DNA in these preparation, the greater part of which was of chromosomal origin. The plasmid DNS enrichment procedure followed the scheme as described in material and methods with the exception, that the ethanol percipitates were recollected in 1,0 ml TES buffer. This 1,0 ml was mixed with the CsCl-EB-gradient solution and the processing and evaluation were carried out as with S. faecalis DT11.

2.2.2. Sucrose gradient centrifugation

The sedimentation studies were performed in 5-20% sucrose gradients in a neutral (pH 7,0) and alkaline (pH 12,0) range. They were carried out using 100 ul of the marker DNA (or TES-buffer) which were mixed thoroughly with 100 ul of the plasmid DNA before being carefully overlayed on top of the 4,8 ml sucrose gradient.

Figure 8 presents the sedimentation profile of the S. faecium 3-0 plasmid. In neutral sucrose the peak is located in the 28th fraction, in alkaline sucrose (all other parameters were held constant) the migration velocity is higher so that the same band is now located in the 21st fraction. The peak is characteristic for CCC DNA.

In figure 9 one observes the sedimentation of the plasmid isolated from S. faecium 3-0 with the ^{14}C-thymidine marked pAM 1. this enables the figuring out of the sedimentation coefficient of pJK3 as indicated in the legend. The value of 22S correlates well with the theoretical value derived from the formula $S_{ccc} = 0{,}034 MW^{0{,}428}$* (Bazaral and Helinski 1968) showing 21,8 S

In the following experiments, the S. faecium 3-0 plasmid was employed as sedimentation marker after undergoing ^{14}C-thymidine labelling. Cultivation of the strain and isolation of plasmid DNA followed the description given for S. faecalis DT11.

* MW = molecular weight of the plasmid concerned, here 3,5 Mdal.

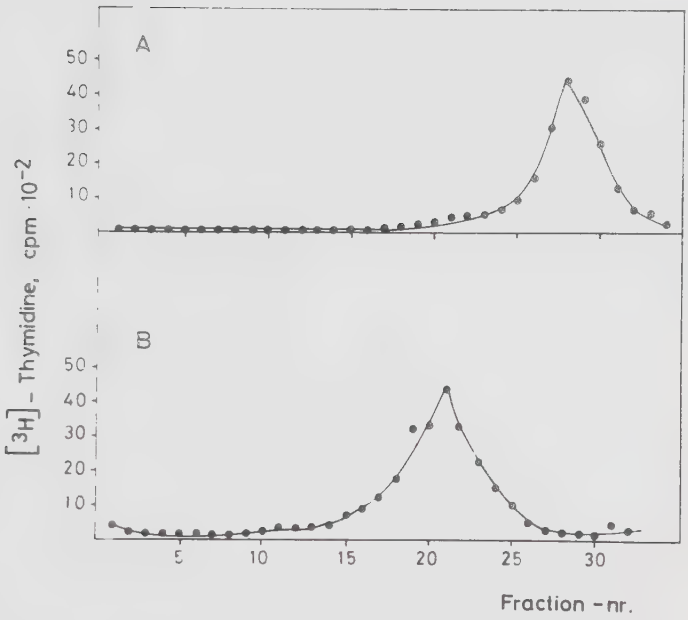

Figure 8: Sedimentation profile of the S. faecium 3-0 plasmid through neutral (A) and alkaline (B) sucrose.

Left part of the graph: tube's bottom; sucrose concentration uptake from left to right. The run was performed at 160000 g and 20°C and 40 min in swing-out-buckets. For evaluation of radioactivity see under material and methods.

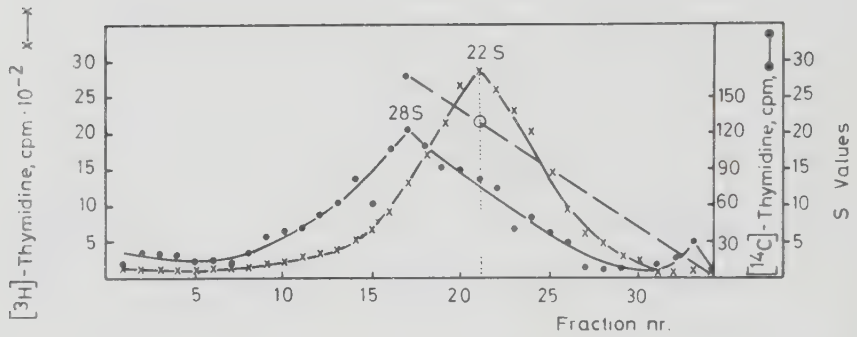

Figure 9: Neutral sucrose gradient of the S. faecium 3-0 plasmid (x-x) cosedimented with the ^{14}C-thymidine marked pAMα1 (28 S). The run was performed at 160,000 g and 50 min. Each fraction consisted of two drops.

The S value was determined in drawing a straight line vertically to the "x" axis (at the top fraction) and drawing on it a scale with S values (from 1-30 for example). This is followed by drawing a line that connects the peak of the known plasmid at the hight of its S value (in accordance with the scale) with the "x" - axis at the point of the last fraction (---). An imaginary point of intersection (●) occurs now at the right hand side of the known peak between this line and the imaginary line that connects the peak of the unknown plasmid with the hereto belonging fraction number (.....). By drawing an imaginary line (parallel to the "x" axis) from this point to the S value skale, we can read the appropriate sedimentation coefficient of the unknown plasmid.

In figure 10 A we observe the sedimentation profile of the S. faecium 25-0 plasmid DNA in neutral sucrose. The first peak (fraction 34) consists of the "mini" and "midi" plasmids (as will be explained in figure 11) the second (fraction 24) of the "maxi" plasmid with its 13 Mdal. The proceeding experiment employed alkaline sucrose (fig. 10 B) as sole altered parameter. The first peak moves a little quicker under the new conditions and is found nr. 32; the second peak has split into two smaller ones. The first of the two consists of OC, the second of CCC plasmid DNA of the "maxi" plasmid. These findings are supported by the electrophoretic migration as shown in fig. 26 and 27 (demonstrating the OC - band only for the "maxi" plasmid).

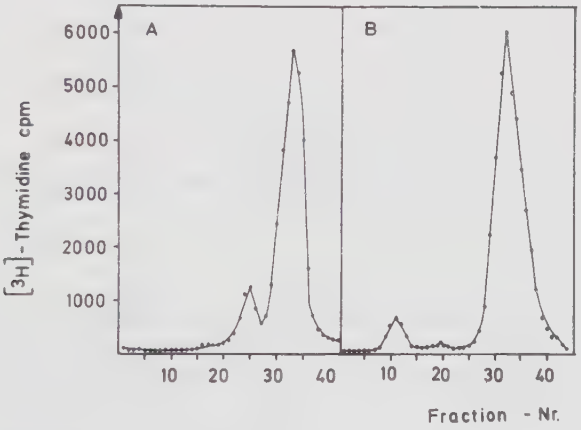

Figure 10: Sedimentation profile of the S. faecium 25-0 plasmid DNA through neutral (A) and alkaline (B) sucrose.

The run was performed at 160,000 g for 40 min (A) and 50 min (B). The tubes were subsequently puncutred and fractions (each 2 drops) collected directly on Whatman nr. 1 filter squares. These were washed and put into scintillation vials for radioactive evaluation.

In figure 11 we observe the cosedimentation of the S. faecium 25-0 plasmids with pJK3 as marker. For the purpose of marking the "mini"

and "midi" plasmids distinguishable from one we collected fractions consisting of 1 drop only causing hereby the "stretching" of the "x" axis. As was observed (and could be reconfirmed in the following) two peaks are found, which overlap one another in their greater part. This is due to the relatively close MW and sedimentation coefficient.

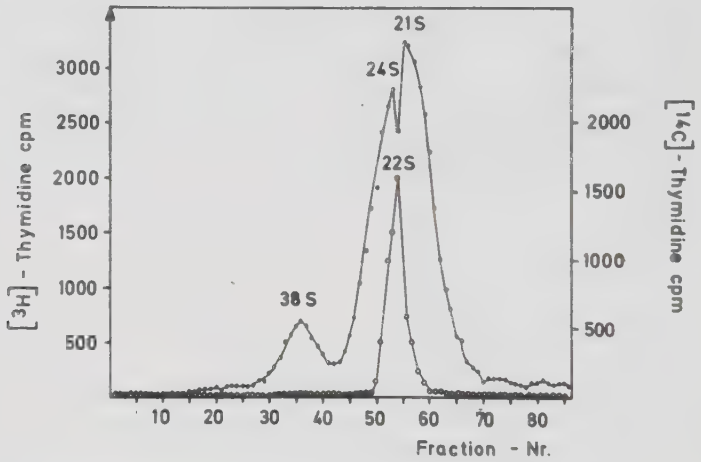

Figure 11: Cosedimentation of S. faecium 25-0 plasmids (•) with the S. faecium 3-0 (o) employed as marker through neutral sucrose. (50 min at 160,000 g and 20°C).

The sedimentation coefficients figured according to the scheme dealt with in the legend to figure 9 are 21S for the "mini", 24S for the "midi" and 38S for the "maxi" plasmid. All values correlate well with the theoretical ones figured with the aid of the formula on page 30 with the one exception, that the "mini" plasmid should have had a calculated value of 19S whereas 21S was experimentally determined.

For the purpose of "revealing" the overlapped OC peak of the "maxi" plasmid this sedimentation was repeated, this time through alkaline sucrose. In figure 12 we see the migration profile. Indeed, as already observed in figure 10, the CCC form of the "maxi" plasmid migrates quicker leaving a smaller peak behind.

Employing the above mode of S value determination, the smaller peak was figured to have a sedimentation coefficient of 29S. The relation between Sccc/Soc is 1,3, a value which agrees well with the relation of Sccc/Soc as a function of MW (13 Mdal in the present case) as described by Clows (1972).

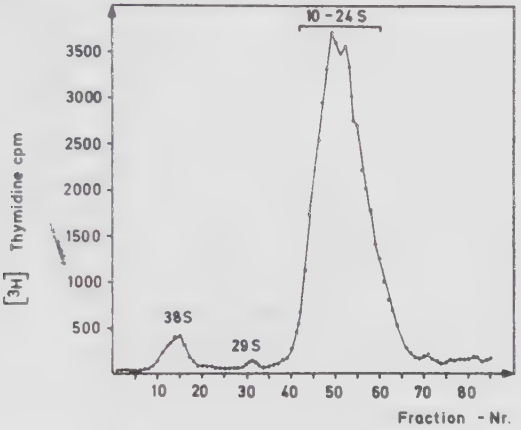

Figure 12: S. faecium 25-0 plasmids sedimented through alkaline sucrose.

100 µl plasmid DNA were mixed with 100 µl TES-buffer and carefully overlayed on top of the gradient. Centrifugation was performed at 160,000 g for 40 min and 20°C. Every fraction consisted of 1 drop and was measured in the scintillation counter for 10 min (rate of failure ± 5%).

3. Plasmid DNA mobilizing experiments

Following the chemical and physical characterization of the S. faecium plasmids as described, our interest was diverted to the biological functions coded by them.
Numerous experiments aimed at transforming the isolated plasmid DNA, employing S. faecium 158 and S. faecalis JH2 as recipients, proved unsuccessful. At this stage our awareness was diverted to the findings of Jacob and Hobbs (1974), Tomura et al. (1973) and Dunny and Clewell (1975) who demonstrated the existance of conjugal transfer of certain plasmids in various strains of S. faecalis. Dunny and Clewell reported, that the hemolysin determining plasmid pAMβ1 can promote its own transfer as well as mobilize a small nontransmissible drug resistance plasmid.
In connection with these reports we decided to take advantage of the self-transferability of plasmid pAD1tc, and try and employ it as mobilizing agent to transfer the S. faecium plasmids into a third strain. Hereby, an isogenic pair, which differs only in the presence or absence of the cryptic plasmid, would have been constructed. (see figure 14).
Plasmid pAD1tc (see figure 13) has a molecular weight of 38 Mdal and its CCC - form shows in neutral sucrose gradient a sedimentation coefficient of 65S. This plasmid codes for the production of a bacteriocin, hemolysin (horse-blood erythrocytes) and the resistance towards tetracycline (MIC 62,5 µg/ml). It had originally been harbored by S. faecalis DS16 (which has a tetracycline resistance determinant located on its chromosome) as pAD1 with a MW of 35 Mdal. During filter mating pAD1 picked somehow the tetracycline resistance marker (transposon) and was hitherto named pAD1tc showin in accordance with its elongation the elevated MW of 38 Mdal.

3.1 Transfer of pAD1tc into S. faecium 25-1 and S. faecium 3-1

Due to the fact that antibiotic resistance pattern of S. faecium 25-0 and 3-0 did not vary from the one of S. faecalis JH2-3 harboring plasmid pAD1tc (pAD1tc was inserted into S. faeclis JH2-3 by means of filter mating as performed by Arth Franke, University of Michigan) - see table 1 - as to allow their selection after

mating, we had to provide these recipients with a marker. For this purpose the antibiotics rifampicin and fusidic acid are most commonly used.

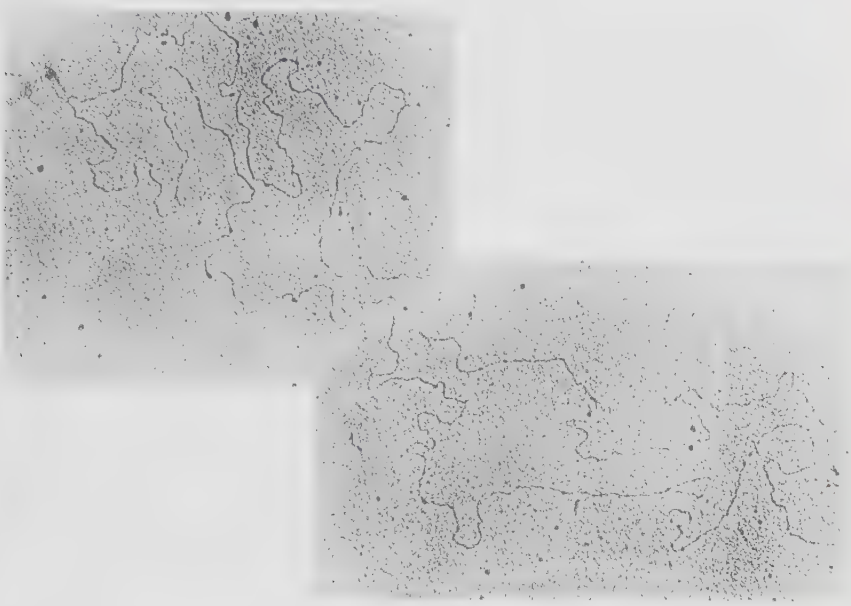

Figure 13: Plasmid pAD1tc isolated from S. faecalis JH2-3. (x 40,000)

Strain JH2-3 harboring this plasmid was cultivated in M9 medium supplemented with 7,5 uCi/ml tritiated thymidine. The cells were harvested at mid-log-phase, lysed with lysozyme/EDTA/sarkosyl+PEG and centrifuged to equilibrium in a dye buoyant density gradient. The plasmid band was than pooled and dialysed against SCC buffer (1,5 mM NaCl and 1,5 mM sodium citrate) for 4 hrs. in the cold until the ethidium bromide diffused away. Subsequently 25 ul hereof were processes for electron microscopic investigation as described in details in material and methods.

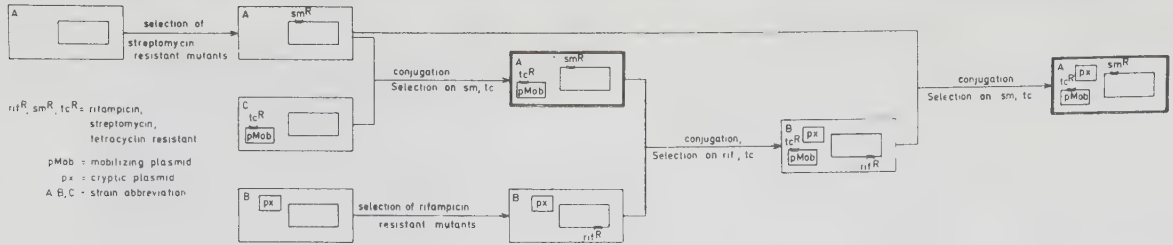

Figure 14: The system illustrates an approach to the revealing of crypticity of plasmids by comparing isogenic pairs (marked by the darker lines). The bacterial cell is schematically drawn as a rectangular, the same is true for the chromosome and the smaller plasmids; genetic markers on either chromosome or plasmids are indicated by bars.

Taking advantage of a mobilizing plasmid (pMob), this system, is aimed at emplowing this characteristic in transferring a cryptic, potentially non-self-transferable mini plasmid into a plasmid free wild strain. This strain's transconjugants differ therefore only in the presence or absence of the cryptic plasmid and can be compared with one another up until the point where some difference in a phenotypical trait occures, pointing at a characteristic coded for by the plasmid in question. The selection for antibiotic resistant mutants (strain A: streptomycin, B: rifampicin) constitutes a basic requirement for the selection of appropriate transconjugants. Strain C is the host of the mobilizing plasmid. Such a plasmid has to possess the following attributes: a. self-transferability, b. known phenotypical traits among which at least one antibiotic resistance marker is a must (here tetracycline). Strain A is the plasmid-free wild strain employed as host for both mobilizing- and cryptic plasmid. It constitutes, in fulfilling this intermediating function, the limbs of the isogenic pair.

3.1.1 Selection for antibiotic resistant recipients

We commenced in preparing TPB-agar containing 25 µg/ml fusidic acid on which 0,1 ml portions (of a 100 fold concentrated 50 ml o.n. culture) of each of the S. faecium strains were streaked. Incubation followed at 37°C for 48 hrs. and the antibiotic resistant mutants were purified again on fusidic acid supplemented TPB-agar in order to determined their stability. A single CFU was than picked up and recultivated in 5,0 ml TPB. After 18 hrs. the cells were harvested and resuspended in 0,5 ml. 0,1 ml portions here of were spread on agar plates containing 25 µg/ml rifampicin (Prior experiments showed, that the spontaneous mutation rate to rifampicin resistance occured much more often then to fusidic acid resistance). Resistant colonies were purified on the same agar as well as on blood agar before choosing one of them for further investigations. The mutant rifampicin- and fusidic acid resistant strains were designated S. faecium 25-1 and S. faecium 3-1. Table 2 shows the minimal inhibition concentrations for both strains.

MIC / Strain	rifampicin	fusidic acid
S. faecium 25-1	2000 µg/ml	1125 µg/ml
S. faecium 3-1	1125 µg/ml	1000 µg/ml

Table 2: Minimal inhibition concentrations (MIC) following selection for antibiotic resistant colonies.

In proceeding the experiments both strains were inoculated with a needle in the center of two petri dishes, incubated o.n. and subsequently killed over chloroform vapor (for details see material and methods). The petri dishes were then pre-heated and each of them was overlayed with 3,0 ml of soft agar containing 0,1 ml of an o.n. culture of: first plate - S. faecium 158 (to provide us with information as to possible alternation in bacteriocinogenity due to antibiotic resistance mutation); second plate: S. faecalis strain JH2-3pAD1tc (to check whether this strain, to be employed as plasmid donor in the following experiments, is susceptible to-

wards the bacteriocin produced by the S. faecium strain).
The results of this experiment showed that:

1. no alternation in bacteriocinogenity occured as a result of the mutation
2. Strain JH2-3pAD1tc showed no susceptibility towards the bacteriocins produced by either strains.

The same experiment was reciprocally repeated, (employing S. faecalis JH2-3pAD1tc as bacteriocin donor and S. faecium 25-1 and S. faecium 3-1 as indicators). The bacteriocin, the production of which is determined by pAD1tc, did not hamper the growth of eather one of the S. faecium strains.

3.1.2 Mating procedure

S. faecium strain 3-1, S. faecium 25-1 and S. faecalis JH2-3pAD1tc were cultivated separately in 5,0 ml M9-medium. After an o.n. incubation the following culture mixtures were prepared:

A. 4,5 ml sterile M9 medium + 0,25 ml S. faecium 25-1 culture + o,25 ml of the S. faecalis JH2-3pAD1tc culture.
B. Same as A with the exception, that S. faecium 25-1 was replaced by S. faecium 3-1.

The controls to this mating experiment consisted of three cell-suspensions each containing 0,25 ml (o.n. cultures) of only one of the strains employed in the above mating mixture and 4,75 ml sterile M9 medium.
Each mixture was thoroughly vortexed and forced through a Millipore membrane filter attached to a 5,0 ml syringe. The filters were subsequently removed and placed on blood agar. Incubation was followed at 37°C for 18 hrs, after which the filters were resuspended in 1,0 ml of the appropriate dilutions were streaked on the agar containing the selective reagents.
Transconjugants of the S. faecium strains were selected on horse-blood-agar containing rifampicin, fusidic acid and tetracycline. The number of donor cells was determined by their selection on tetracycline and streptomycin containing horse-blood-agar. The S. faecium controls were streaked on horse-blood, tetracycline-abd streptomycin supplemented agar; S. faecalis JH2-3pAD1tc was streaked on rifampicin and fusidic acid supplemented agar.

Final evaluation of the plates was carried out after 48 hr. incubation at 37°C.
Frequency of transconjugant formation was 10^{-7} fir S. faecium 3-1pAD1tc and $1,6 \cdot 10^{-7}$ per recipient cell for S. faecium 25-1pAD1tc. In order to determined possible plasmid segregation both sorts of transconjugants went through 40 generations of growth on the selective agar.
Figure 15 showes the growth of S. faecium 25-1 (left half) and S. faecium 25-1pAD1tc on horse-blood supplemented TPB-agar. The production of ß-hemolysin by the transconjugant is evident.

Figure 15: S. faecium 25-1 (left) and S. faecium 25-1pAD1tc streaked on horse-blood supplemented TPB-agar and incubated for 24 hrs. at 37°C .

The MIC of tetracycline towards the S. faecium transconjugants and the donor strain S. faecalis JH2-3 pAD1tc was tested in tryptose phosphate broth and showed uniformly 125 µg/ml.
The production of ß-hemolysin towards horse-blood-erythrocytes proved to be dependent on the culture's age in both donor- and recipient strains. An aging (i.e. o.n.) culture produced a 100% hemolysin positive CFUs while only 10% of the total CFU (derived from cultures in their mid-logarithmic phase of growth) were surrounded by zones of hemolysis. Usually, evaluation was carried out after 18 hrs. of incubation.
According to our observations, the ability to produce hemolysin was presented in every one of the CFU, its phenotypic expression, however, is repressed in the earlier stages of growth. Of the hemolysin negative CFU, all reverted to hemolysin producers after prolonging the incubation for additional 12 hrs.

For the purpose of determining co-transfer of bacteriocin production (as coded by the pAD1 part of the plasmid), we searched for indicator strains which were resistant towards the bacteriocins produced by S. faecium 3-1 and 25-1 and sensitive towards the ones produced by the transconjugants. S. faecium strain 38 and 160 as well as S. faecalis strain 202 and 214 fulfilled the requirements concerning S. faecium 25-1 and its derivative. All other strains tested as indicators proved susceptible towards the bacteriocin produced by this strain and were therefore excluded from the following test assay.

Indicator / Donor	S. faecium strain		S. faecalis strain	
	38	160	202	214
S. faecium 25-1	-	-	-	-
S. faecium 25-1 pAD1tc	11 mm	10 mm	10 mm	11 mm
S. faecalis JH2-3	-	-	-	-
S. faecalis JH2-3 pAD1tc	2 mm	3 mm	3 mm	2 mm

Table 3: Susceptibility of different enterococcal strains towards the bacteriocin produced by the strains which are enlisted under "donors". The data represent inhibition-zone-diameter. (See text for experimental specifications)

The bacteriocin assay was carried out on TPB-agar. The donor strain(s), (six at a time, clockwise on a petri dish) were inoculated with preautoclaved toothpicks, incubated o.n. and subsequently killed over chloroform vapor. 0.1 ml of the indicator strain o.n. culture (TPB) were mixed with 3,0 ml 0,7% TPB-agar and carefully overlayed on the prewarmed (37°C) ground layer. Evaluation (i.e. measurement of inhibition-zone-diameter) followed after 18 hrs of incubation.
The results presented in table 3 show, that S. faecium 25-1 pAD1tc gained the ability to produce a new bacteriocin compareable to the one produced by S. faecium 25-1. The observed inhibition-zone diameters vary insignificantyl amongst the indicator strains.
It seems however, that we are confronted with a synergistic mechanism concerning the bacteriocin produced by the transconjugant. Whereas the bacteriocin produced by the donor strain S. faecalis JH2-3 pAD1tc causes only minimal inhibition of growth and the one produced by the S. faecium 25-1 does not interfere with the indicator strain at all, a fusion of both bacteriocin in one strain arises significantly its bactericidal efficiency.

Although 65 different enterococcal strains were screened as to their susceptibility towards the S. faecium 3-1 and the transconjugant S. faecium 3-1 pAD1tc bacteriocin, all of them proved sensitive towards the bacteriocin produced primerily by S. faecium 3-1. Hence, for the time being, no tool to keep it apart from the pAD1tc determined bacteriocin is available.
The transfer of hemolysin production and tetracycline resistance was evidenced by growth on appropriate selective agar as already shown in figure 15.

3.2. Reciprocal mating attempts: S. faecium JH2-4 as recipient

According to the findings of Van Embden an Cohen (1973), Jørgensen et al. (1976), Williams et al (1977) and Clewell et al. (1976), nonconjugative plasmids can be transferred to other bacteria from hosts harboring a conjugative plasmid.
Due to the fact, that we had no criterion to judge the transferability of the cryptic S. faecium plasmids (doubtful as far as "mini" plasmids are concerned because according to the models

existing, the MW of resistance transfer factors for example is 16,56 Mdal. This according to Perlman and Round's publication from 1975), neither phenotypical traits to select for, we chose plasmid pAD1tc as mobilizing vector.
The following conjugation experiments were aimed at transferring pAD1tc and hitherto the S. faecium plasmids from the transconjugants back to the wild strain S. faecalis JH2.
Prior to this procedure we had to select for an S. faecalis JH2 colony, with an antibiotic resistance marker other then streptomycin. This because both S. faecium strains dealt with here are resistant to 1000 µg/ml (MIC 1125 µg/ml) of this substance. Our choice fell on erythromycin because of the very low rate of spontaneous resistance acquired towards this antibiotic.

0,5 ml of S. faecalis JH2 o.n. culture in TPB were poured into 100 ml TPB supplemented with 3,5 µg ethidium bromide. After 6 hrs of incubation the cells were pelleted and recollected in 1,0 ml. of sterile broth (approx. 10^{11} cells). 0,1 ml of this suspension was streaked on TPB agar containing 10 µg/ml erythromycin. The erythromycin was suspended in methanol and poured into the autoclaved medium which was kept at 45°C in a warer bath. The methanol evaporated at once. A 48 hrs incubation at 37°C followed before evaluation of resistant colonies was made. Two were isolated. One, with an MIC of erythromycin of 1250 µg/ml (as determined subsequently in TPB) was picked up for the ongoing experiment. (Designation: S. faecalis JH2-4).

The mating system aimed at constructing isogenic pairs, consisted of S. faecalis JH2-4 as recipient and either S. faecium 25-1 pAD1tc or 3-1 pAD1tc as donor.

5,0 ml o.n. cultures of each of the above mentioned strains were prepared and 0,25 ml thereof added to the mating mixture, which also contained 4,5 ml M9 medium. A total of 5,0 ml was forced through a syringe to the tip of which we attached a filter. The filter was placed on blood agar and incubated overnight. The filter was then resuspended in 1,0 ml of M9 medium of which 0,1 ml. portions were streaked on horse-blood-agar supplemented with tetracycline and erythromycin (each 10 µg/ml). Evaluation followed

after 48 hrs. of incubation to allow optimal phenotypic expression.

3.2.1 Outcome of the mating: S. faecium 25-1 pAD1tc as donor

The rate of transconjugant's formation for S. faecalis strain JH2-4 pAD1tc(25) was $5 \cdot 10^{-5}$ per recipient. Hemolysin production varied depending on the CFU's age. MIC of tetracycline tested for 10 of 100 isolated transconjugants was 125 ug/ml, this was identical with the parental strain. To check bacteriocin transfer we used the same four indicator strains employed for the S. faecium 25-1 pAD1tc transconjugants. The test was carried out on TPB-agar applying the chloroform method.
The results (see also table 4) pointed out, that 98 of the isolated transconjugants with the presumable phenotype of S. faecalis JH2-4 pAD1tc(25) reacted exactly like S. faecalis JH2-3 pAD1tc and therefore it was clear that no genetic determinants, carrying information that dealt with bacteriocin production, had been exchanged.

Indicator / Donor	S. faecuim strain		S. faecalis strain	
	38	160	202	214
S. faecium 25-1	-	-	-	-
S. faecium 25-1pAD1tc	9 mm	10 mm	11 mm	11 mm
S. faecalis JH2-4	-	-	-	-
S. faecalis JH2-3pAD1tc	2 mm	2 mm	-	2 mm
S. faecalis JH2-4pAD1tc(25)1	10 mm	13 mm	10 mm	9 mm
S. faecalis JH2-4pAD1tc(25)2	9 mm	10 mm	11 mm	8 mm

Table 4: Susceptibility of different enterococcal strains towards the bacteriocin produced by S. faecium 25-1, S. faecalis JH2-4 and their transconjugants. "-" means no inhibition of growth. The numbers in mm express inhibition zone diameter.

According to the findings represented in the above table, the last two strains in the list show the same synergistic phenomenon described in connection with the S. faecium 25-1 pAD1tc transconjugant. It is obvious that the bacteriocinogenic substance produced by S. faecalis JH2-3 pAD1tc is much less effective (in terms of

inhibition-zone diameter) than the one produced by S. faecium 25-1 pAD1tc or S. faecalis JH2-4 pAD1tc(25)1 or 2. In fact, it seems that the latter two react in exactly the same way towards the indicator strains.

The results however, were reproducanle fpr two generations only. In the thirs generation, these newly constructed transconjugants reacted in the same way as S. faecalis JH2-4 pAD1tc, i.e. produced merely "mini" zones of inhibition in indicator lawns. (Diameter: 1-2 mm). These findings could point out a segregation of the transferred plasmids occuring during consecutive cell devisions.

The next step was to compare the sugar utilization spectrum of the transconjugants with their parental strains.

For this test, CTA was employed as basal medium. 5,0 supplemented with the appropriate sugar (as sole carbon source) were inoculated with 0,05 ml of an o.n. culture of the strain to be tested, incubated at 37°C and checked every 24 hrs. (for a total of 8 days) for acid building.

The following sugars were utilized by both donor- and recipient strains (and hitherto transconjugants): starch, mannose, trehalose, lactose, cellobiose, glucose, raffinose, aesculine, salicine, galactose, sucrose and dextrin.

Differences in their utilization spectra occured with the following:

Strain / Carbohydrate	S. faecium 25-1	S. faecalis JH2-3pAD1tc
Rhamnose	-	+
Glycerol	-	+
Glycogene	-	+
Malonate	-	+

Table 5: The differences concerning carbohydrate utilization between S. faecium 25-1 and S. faecalis JH2-3pAD1tc.

All S. faecalis JH2-4pAD1tc(25) transconjugants showed the same reaction as JH2-3pAD1tc and were therefore considered to be S. faecalis JH2-4pAD1tc transconjugants, that did not take part

in exchanging genetic material originating from S. faecium strain 25-1 pAD1tc (with the exception of pAD1tc). This is true only for the above tested features.
Plasmid isolation (with the aid of CsCl-EB-density gradient centrifugation) from S. faecalis JH2-4 pAD1tc(25) 1, 2 and 10 randomly picked transconjugants, failed to produce plasmids originating from S. faecium 25-1 (electron microscopic investigation).

3.2.2 Outcome of the mating: S. faecium 3-1pAD1tc as donor

0,25 ml of an o.n. culture of S. faecium 3-1 pAD1tc in M9 medium were mixed with the same amount of S. faecalis JH2-4 culture + 4,5 ml M9-medium and forced through a Millipore filter as indicated in material and methods. The same procedure was undertaken with 0,25 ml of each one of the mating partners, being separately mixed with 4,75 ml M9. (control probes). The filters were subsequently pressed on blood agar plates, incubated o.n. and each resuspended in 1,0 ml of M9 broth. Transconjugant's selection took place on erythromycin and tetracycline (10 µg/ml each) supplemented TPB agar. The Nr. of donors originating from the mating mixture was determined on rifampicin and fusidic acid (20 µg/ml each) containing agar, the Nr. of recipients plus transconjugants on erythromycin supplemented agar. The donor- and recipient control charges were each streaked on tetracycline and erythromycin supplemented agar. Incubation was carried out for 48 hrs in order to allow for phenotypic expression of resistance. The evaluation showed that there was no growth of either control charges i.e. no spontaneous mutation neither to erythromycin (S. faecium 3-1 pAD1tc) nor to tetracycline resistance (S. faecalis JH2-4). The number of transconjugants was found to be $6 \cdot 10^7$/filter disc. The total number of recipients and transconjugants, as determined on erythromycin supplemented agar, was $1,6 \cdot 10^8$ (net number of recipients 10^8/filter disc). These data suggest a conjugation rate of 1:1,6 per recipient.
100 transconjugant's CFU were repurified on horse-blood, erythromycin and tetracycline supplemented agar and subsequently tested as to their bacteriocinogenity.
As mentioned in connection with the construction of S. faecium

strain 3-1 pAD1tc, the bacteriocin primerily produced by this strain has a very broad inhibitory spectrum towards enterococci. If the production of this plasmid should be plasmid determined, one should find a number of S. faecalis JH2-4 pAD1tc(3) transconjugants, with the same spectrum. We picked six indicator strains in order to test for bacteriocin susceptibility, All were resistant to the bacteriocin coded for by pAD1tc.

Indicator / Donor		S. faecium strain				S. faecalis	
		38	59	160	158	214	160
S. faecium	3-1	22 mm	21 mm	13 mm	22 mm	13 mm	13 mm
	3-1pAD1tc	20 mm	23 mm	14 mm	21 mm	13 mm	14 mm
S. faecalis	JH2-1pAD1tc	-	-	-	-	-	-
	JH2-4pAD1tc(3)1	11 mm	9 mm	9 mm	10 mm	8 mm	9 mm
	JH2-4pAD1tc(3)2	10 mm	4 mm	10 mm	12 mm	5 mm	10 mm
	JH2-4pAD1tc(3)3	12 mm	12 mm	12 mm	15 mm	13 mm	12 mm
	JH2-4pAD1tc(3)4	-	-	-	-	-	-

Table 6: comparative studiey of bacteriocin susceptibility following the transfer of pAD1tc and apparently pJK3 from S. faecium 3-1 to S. faecalis JH2-4. for details see text. Numbers point inhibition zone diameter. "-" means no inhibition.

The results presented in table 6 poit out, that 3% of the examined S. faecalis JH2-4 transconjugants reacted in the same manner as S. faecium 3-1 did in respect to the bacteriocin produced by them. This is in cotrast to S. faecalis JH2-1 pAD1tc, whose bacteriocin remained inefficient. With S. faecalis JH2-4pAD1tc(3)4 we brought an example representing the 97 remaining transconjugants tested in this manner. As far as their bacteriocin is concerned, they all reacted as colonies, derived from S. faecalis JH2-4pAD1tc and showed no evidence of having been involved in genetic transfer processes with S. faecium 3-1.

The results presented here were reproducible for one generation, after which bacteriocin production against the indicator strain ceased. We were already confronted with this phenomenon when

trying to determine the phenotypic traits of the S. faecium 25-1 plasmids (S. faecalis JH2-4pAD1tc(25) - transconjugants). The results prove however, that together with pAD1tc, DNAcoding for the S. faecium 3-1 bacteriocin had been cotransferred. The transfer affected most probably the S. faecium "mini" plasmid because, as an autonomous replicon, the chances for its transfer to become abortive are much higher. This compared with the exchange of genetic material in the chromosomal level, where replication is guaranteed through the already existing host mechanisms.

3.3 Electrophoretic analysis of the transconjugant's genotypes

Apart from giving a phenotypical presentation to the transconjugants S. faecium 25-1pAD1tc and 3-1pAD1tc, our aim was to provide proof for the presence of genetic material in the recipient strain i.e. to employ a method, with which aid the cell-plasmids can be physically visualized. The vertical slab gel electrophoresis technique has been most successfully employed for the purpose of demonstrating the pkasmid content of bacterial cells. The method, first employed for this very purpose by Meyers et al. (1976), aids in distinguishing the different teritery structural forms of plasmids (linear, open circular and covalently closed circular).

DNA preparation: 70 ml M9 medium (supplemented with tetracycline for the pAD1tc plasmid carrying strains) were inoculated with 0,7 ml of an o.n. culture of the following strains: S. faecalis JH2, S. faecalis JH2-3 pAD1tc, S. faecium 3-1 and 3-1 pAD1tc as well as S. faecium 25-1 and 25-1 pAD1tc. The cultures were incubated at 37°C until $\Delta E_{550\ nm}$ = 0,80 (approx. 6-7 hrs), centrifuged, and the pellet was recollected in 1,5 ml of TES buffer supplemented with sucrose (200 mg/ml). After the addition of lysozyme (1,5 ml 6 mg/ml) and EDTA (1,5 ml 0,25 M pH 8,0) the spheroplasts were lysed by adding a mixture of sarkosyl/PEG. The cleared lysate was frozen (-20°C) until the following day. Thawing of the preparate was followed by intensive shearing and subsequent dilution to a total of 70 ml with TES-buffer. We proceeded with alkaline denaturation followed by renaturation with Tris-HCl after which NaCl was added (total 3% W/V) followed by 0,5 Vol of phenol. Deproteinization was followed by ethanol percipitation of DNA.

The percipitates were pelleted in the ultra centrifuge after 48 hrs at -20°C, They were recollected in TES-buffer to form a concentration of 1 µg/ml DNA (see material and methods for quantitative determination procedure).

For electrophoresis, Seakam agarose was dissolved (1,0%) in boiling borate buffer. The slab gel was was formed while the agarose still showed a temperature of greater than 90°C.

25 µl of each of the above DNA preparations were mixed with the same amount of dye solution and electrophoresis was started with 1 hr. at 120 V/35 mA followed by 22 hrs. at 20 V/4 mA.

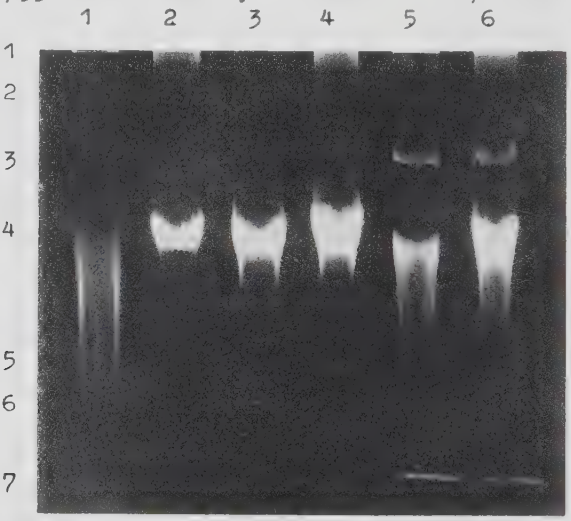

Figure 16: Electrophoresis of the enterococcal strains as specified below, showing the plasmid DNA patterns of donor- and recipient (transconjugants) strains. From left to right: S. faecalis JH2-3, S. faecalis JH2-3pAD1tc, S. faecium 3-1 and 3-1pAD1tc, S. faecium 25-1 and S. faecium 25-1pAD1tc.
1. starting point 2. pAD1tc with a MW of 38 Mdal, visible in 2nd, 4th and 6th slot 3. "maxi" plasmid harbored by S. faecium 25-1, MW 13 Mdal 4. chromosomal band (all slots) 5. "midi" plasmid from S. faecium 25-1, MW 4,7 Mdal 6. plasmid pJK3, MW 3,5 Mdal and 7. the "mini" plasmid from S. faecium 25-1, MW 2,4 Mdal.

Earlier experiments with the electrophoresis aparatus used by us proved, that a 1 hr. run at a relatively high voltage is necessary to allow plasmid DNA with a MW greater than 25 Mdal enter the gel. (In this run we were dealing with pAD1tc with a MW of 38 Mdal).

After the run came to an end, the gel was placed in a dye solution for 20 min. (1 µg/ml ethidium bromide in distilled water). The gel was observed subsequently under short wave UV light and photographed with a polaroid-type camera.

In correlation with the phenotypic features of the pAD1tc harboring transconjugants, the band representing this plasmid (see nr. 1 in figure 16) is visible in all three of them. As repeated runs proved, the intensity of the UV fluorescence of the pAD1tc band is in all three transconjugants much less intensive than the other plasmids, pointing at a relatively small copy number of this plasmid per cell. (Plasmid replication under stringent control). Furthermore, with the phenol enrichment method only covalently closed circular plasmid DNA is observed. Open circular DNA would band separately and migrate much slower as observed by Meyer et al. 1976. The conversion to the OC form is practically brought to a halt if samples are frozen in TES-buffer at -20°C immediately after collection of ethanol percipitates. This fact constitutes an important asset especially in screening plasmids of wild strains. The band representing chromosomal DNA consists of linear debris resulting from the intensive shearing and the subsequent alkaline denaturation. The average size of these fragments is homologous. The very diffuse bands observed at slots nr. 2,3 and 4 at the level of the "maxi" plasmid harbored by S. faecium 25-1 are unspecific and give the distance of migration of the DNA samples during the first hour (120 V). This conclusion was drawn after measuring the distance of migration of the dye (with which the DNA samples were mixed) just before changing over to 20V.

4. Evidence for plasmid coded bacteriocin production

4.1 Mating experiment between S. faecalis JH2-2 and S. faecium 3-0

For the purpose of eventually conferming the results we described in the previous page, a direct mating between S. faecalis JH2-2 and S. faecium 3-0 was examined. Both strains were grown in 5,0 ml M9 medium. 0,25 ml of each culture were mixed with 4,5 ml M9 medium . For (negative-) control purposes, 0,25 ml from the S. faecalis JH2-2 culture were separaetly mixed with 4,75 ml M9 medium. In both cases the culture solutions were forced through a Millipore filter, each filter placed on blood agar plates and incubated for 18 hrs. This was followed by resuspending the filter in 1,0 ml broth. We continued in carrying out serial dilutions of which the 10^{-5} and 10^{-6} were streaked on TPB agar supplemented with rifampicin and fusidic acid (20 ug/ml each to select for S. faecalis JH2-2).

Following an incubation for 24 hrs, the petri dishes were replica plated on antibiotic free TPB agar (to allow subsequent indicator growth) with the aid of velvet cloth and reincubated for an additional 24 hrs. The CFU growing on the antibiotic free medium were killed over chloroform vapor and overlayed with 3,0 ml of TPB soft-agar containing 0,1 ml of an S. faecium 158 o.n. culture. The same procedure was performed with the control batch (consisting of S. faecalis JH2-2). Evaluation could be performed within 6 hrs of incubation. 4,5% of S. faecalis JH2-2 CFU (i.e. 41 CFU out of a total of 901) produced a zone of inhibition in the indicator lawn. Upon following these CFU back on the "master" plate and reperforming the bacteriocin test, none retained the ability to produce it. This phenomenon reproduces the results achieved in the previous experiments. It hints furthermore at the fact, that transfer of bacteriocin production, takes place without an intermidiating action of a mobilizing plasmid as was assumed to the very day for "mini" plasmids.

4.2 Characterization of the phenotypical traits coded by S. faecium M16 plasmid DNA

As the previous experiment showed, we could emonstrate the exchange of genetic material between S. faecium 3-0 and S. faecalis JH2-2. What we lacked was a detectable manifestation of the phenotypical traits concerned. The new characteristics were reproducible for only one to two generations (abortive conjugation).
We decided to introduce S. faecium M16 as plasmid recipient, because the chances for plasmid compatibility within this strain could prove to be greater. This in view of the fact that donor and recipient are of the same genus.

S. faecium M16 has been isolated from patient's material and been identified as such by Kühnen (1980). The "M" stands for multiple antibiotic resistance (MIC of tetracycline: 320 ug/ml, erythromycin: 256 ug/ml). The strain was shown by the above author to harbor a plasmid which is 9,2 u long (corresponding to a MW of 19,1 Mdal) and is non-bacteriocinogenic (according to the detection methods employed by us).
Our first aim in investigating this strain was to try and find out whether this plasmid contains an R-factor and whether it is self-transferable.

4.2.1 Mating experiment between S. faecium M16 and S. faecalis strain JH2-2

O.n. cultures (5,0 ml M9 medium) were prepared from S. faecium M16 and S. faecalis JH2-2. 0,25 ml of each were mixed in 4,5 ml M9 and forced through a Millipore filter. The filter was then placed on blood agar and after 18 hrs of incubation at 37°C, resuspended in 1,0 ml M9. Selection of transconjugants was carried out on the following media:

1. rifampicin, fusidic acid and tetracycline supplemented agar (the first two in order to select for S. faecalis JH2-2 and the third as probable plasmid marker).
2. rifampicin, fusidic acid and erythromycin supplemented agar (here erythromycin was employed as probable plasmid marker)

3. rifampicin, fusidic acid, tetracycline and erythromycin supplemented agar.

The total nr. of recipient strain's CFU was determined by streaking 0,1 of the 10^{-6} and 10^{-5} dilutions on TPB agar supplemented with rifampicin and fusidic acid. Controls to exclude spontaneous resistance mutation towards erythromycin or tetracycline were performed with the S. faecalis JH2-2 culture alone.

The evaluation carried out 48 hrs later (to allow phenotypic expression) produced only transconjugants on the rifampicin, fusidic acid and tetracycline supplemented agar. As far as the control tests are concerned, no spontaneous mutation towards either antibiotics occured. The rate of tetracycline resistance transfer was found to be 10^{-7}. 3 of the CFU that had gained this trait were subsequently isolated.

For the purpose of determining whether the resistance transfer towards tetracycline coincided with the transfer of the M16 plasmid we performed the following plasmid enrichment procedure:

O.n. culture (5,0 ml M9) were prepared of S. faecium M16, the three isolated, tetracycline resistant S. faecalis JH2-2 derivatives and S. faecalis JH2-2. The growth medium for the S. faecalis JH2-2 derivatives and for S. faecium M16 was supplemented with tetracycline (10 µg/ml). Main cultures were inoculated in the following morning (each 50,0 ml) and after a mid log phase of growth was reached, the cells were harvested and lysed. Plasmid DNA enrichment followed the alkaline denaturation and phenol extraction method.

Ethanol percipitation was carried out for 48 hrs and the DNA pelleted subsequently at 100,000 g, -5°C and 45 min. The pellet was resuspended in TES buffer and devided into 25 µl portions each containing 1 µg/ml DNA. (A preparation produced usually 250-300 µg DNA)

4.2.2 Electrophoretic investigation of the mating product

20 µl DNA of each one of the strains as enlisted above were mixed with 10 ul of a dye solution consisting of brome phenol blue, SDS and glycerol and filled subsequently into the slots form in the electrophoresis gel. The upper buffer chamber of the electropho-

resis apparatus was then filled with borate buffer. Electrophoresis was carried out at 120V/35 mA until the dye front had penetrated the gel (approx. 15 min), and then switched over to 20V 4 mA. After dismounting the gel it was placed in 1 liter H_2O supplemented with 1 mg EB. This dye intercalates with the DNA and reflects UV light at 254 nm. After making the migration pattern visible, it was photographed with a polaroid type camera.
As pointed out in figure 17, the transfer of the resistance determinants towards tetracycline did not (as expected), coincide with the transfer of the S. faecium M16 plasmid DNA. pM16 seems to be nonconjugative or at least noncompatible with the S. faecalis strain. What we are confronted with is genetic transfer at the chromosomal level. It seems furthermore, that the erythromycin resistance is in this case plasmid determined; for the purpose of confirming this hypothesis pM16 had to be eliminated.

Figure 17: Electrophoretic migration pattern of (from left to right) S. faecalis JH2-2, S. faecalis JH2-2 tetracycline resistant derivative and S. faecium M16.

4.2.3 Elimination of the S. faecium M16 antibiotic resistance

According to prior findings (Jacob et al, 1975, Fuchs et al. 1974, Tomich et al, 1976), bacteria maintained for a longer period of time in slant cultures (>6 months) tend to loose their plasmids.

this occures especially if selection pressure assuring plasmid maintenance is absent. (i.e. adding the antibiotic towards which the strain shows resistance to its growth media).
1,0 ml of physiological NaCl solution was pipetted into a 6 month old slant culture of S. faecium M16 (which was kept in the refrigerator). After vortexing, 0,1 ml of the $5 \cdot 10^{-7}$ dilution of this cell suspension was streaked on TPB-plates. This was done in order to allow for growth of 20-30 CFU per petri dish. An o.n. incubation followed after which the so called "master plates" were replica plated on erythromycin (10 µg/ml) supplemented agar. The plates were reincubated and the evaluation of the daughter plates was carried out 48 hrs later. Out of a total of 2543 CFU, 71 could not grow on the antibiotic supplemented agar, 17 CFU regained their resistance in the second passage. (i.e. reinoculated from the master plates onto antibiotic supplemented agar). There was altogether a 2,1% loss in antibiotic resistance. The remaining 54 CFU were now streaked on tetracycline supplemented agar. All retained their resistance towards this antibiotic.
For examination of their plasmid content we picked 10 of these erythromycin sensitive derivatives.

4.2.2 Electrophoretic analysis of the antibiotic resistance cured S. faecium M16 derivatives

Plasmid enrichment procedure followed the alkaline denaturation-phenol extraction procedure. DNA ethanol percipitates recollected following ultra centrifugation (100,000 g 45 min) were portioned in mini plastic tubes each containing 20 ug DNA and frozen at -20°C until further investigations were undertaken.
For electrophoretic investigation of the samples, S. faecium M16 plasmid DNA was applied as positive control.

The results arising from the migration pattern as shown in figure 18 (the eight remaining erythromycin sensitive S. faecium M16 derivatives showed the same pattern) point out, that plasmid pM16 is responsible for the erythromycin resistance. The missing pM16 band within the still tetracycline-resistant S. faecium M16 derivatives providesfurther evidence that this resistanca determinant is located on the bacterial chromosome.

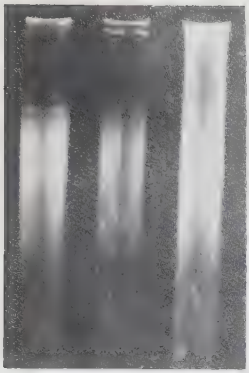

Figure 18: Electrophoretic migration of ethanol percipitated DNA isolated from S. faecium M16 (center slot) and two of its erythromycin sensitive derivatives (to the left and right of this slot). The upper band (center) represents pM16; the band in the upper half of the gel seen in all slots represents chromosomal DNA.

The electrophoresis was run at 120 V for 10 min. (until penetration of dye solution into the gel was clearly visible. This was followed by 22 hrs at 20 V.

4.3 Mating experiment between S. faecium 3-0 and S. faecium M16

After having characterized the antibiotic resistance located on the plasmid harbored by S. faecium M16 we proceeded with employing this strain as plasmid recipient in a mating experiment with S. faecium 3-0. Before doing so it had to be determined whether or not S. faecium M16 is sensitive towards the bacteriocin produced by S. faecium 3-0 and vice versa.

Both strains were inoculated by means of a tooth pick in the center of TPB agar plates and in 5,0 ml TPB broth. After an o.n. incubation at 37°C the CFU were killed with a drop of chloroform and overlayed with 2,5 ml soft agar (0,7%) containing 0,1 ml of the o.n. culture . This was followed by a 6 hrs incubation period after which the indicator lawn was easily detectable.

The evaluation of this test proved that the bacteriocin produced by S. faecium 3-0 inhibited the growth of S. faecium M16 (inhibition-zone-diameter: 18 mm) and that, on the other hand,

S. faecium M16 did not hamper in any way the growth of S. faecium strain 3-0.

As previous experiments hinted at, there were good chances that the plasmid harbored by S. faecium 3-0 codes for bacteriocin production. Mating experiments produced abortive exconjugants, all of which were selected using bacteriocin production as selection marker. S. faecium 3-0 and S. faecium M16 were each inoculated into 5,0 ml M9 medium and incubated o.n. The mating mixture prepared on the following morning consisted of 0,1 ml of the S. faecium 3-0 culture, 0,4 ml of the S. faecium M16 culture and 4,5 ml M9 medium. After intensive vortexing the cell suspension was forced through a Millipore membrane filter . The filter was subsequently pressed on the surface of blood agar and incubated for 18 hrs. This step was followed by removing the filter and vortexing it in 1,0 ml of M9 broth. Selection for the recipient strain took place on erythromycin/tetracycline (10 ug/ml each) supplemented agar . The 10^{-5} and 10^{-6} dilution of the mating mixture were streaked on to it. (0,1 ml). An o.n. incubation followed after which the colonies were replica plated on antibiotic free agar (to allow growth of the indicator strain S. faecium 158). This step was proceeded by another o.n. incubation after which the replica plates were turned over chloroform to kill the colonies. They were subsequently overlayed with 2,5 ml of TPB-soft agar containing 0,1 ml of a S. faecium 158 6 hr. culture.

The evaluation carried out 6 hrs later (first passage) showed that 8,5% (94 colonies out of a total of 908) of the recipient strain gained the ability to produce bacteriocin. The controls set for S. faecium M16 with no mating partner did not produce bacteriocin positive colonies.

The bacteriocin positive S. faecium M16 derivatives were followed back on the master plate, repurified on blood agar and erythromycin/tetracycline agar and reinoculated on TPB agar for reproducibility testing. 24 hrs later the colonies were killed over chloroform vapor (see material and methods for details) and subsequently overlayed with soft agar containing the same indicator strain as above. Evaluation showed that merely 1,2% of the total recipients proceeded in producing bacteriocin. The third passage left us with 5, the 4th with 3, the 5th with two and the 6th with

one S. faecium M16 colony, which acquired the ability to produce bacteriocin. In fact, as following experiments showed, this colony was capable of producing it for 40 generations with repeated reproducibility. Every bacteriocin test was performed by randomly picking a single CFU (always 18 hrs old) as donor derived from the bacteriocin positive transconjugant. All this was undertaken in order to guarantee the selection of a stable transconjugant.

4.3.1 Differentiation parameter between S. faecium M16 and 3-0

Before proceeding with the examination of the bacteriocin produced by S. faecium M16, we decided to introduce some test procedures which will allow for perfect differentiation between the mating partners concerned.
The way in which the colony morphology on blood agar among the strains, was the first mode of differentiation applied. S. faecium M16 produced much bigger CFU's (18 hrs of incubation at 37°C) as did S. faecium 3-0, whose CFU's had almost the size of pin points.
In order to compare suger utilization spectra, both donor and recipient and 10 of the ex-conjugants as well (of which only one maintained the capability of producing the bacteriocin) were inoculated into 5,0 ml nutrient broth supplemented with 1,0% of the carbohydrate to be tested. An o.n. incubation at 37°C followed after which the indicator (brome phenol purple) changed to yellow in the case when acid was produced.
The results showed that S. faecium 3-0 could utilize sucrose. All the remaining strains were sucrose negative (after a total of 8 days of incubation). The antibiotic resistance pattern of the S. faecium M16 ex-conjugants also pointed out at the recipient strain.

4.4 Analysis of the plasmid DNA content of the mating partners and the transconjugant

Following the selection for bacteriocin positive S. faecium derivatives, after the mating procedure with S. faecium 3-0, it was of profound interest, to determined whether the transfer of this feature coincides with the transfer of plasmid DNA.
Similar phenomena (employing other transfer systems) were already described for Col E1 (Arber et al. 1978) and the Clo DF plasmid

(Ionesco et al, 1976) among others.
The strains investigated in the framework of this experiment were, apart from donor and recipient, also the five S. faecium M16 derivatives, which, following the mating, produced bacteriocin for 1 to 5 generations and then ceased to do so.
The strains were each cultivated in 75 ml M9 medium till ΔE_{550nm} = 0,85, subsequently centrifuged and lysed in hypertonic sucrose solution supplemented with lysozyme, (20 min) EDTA (20 min) and a detergent mixture of sarkosyl/PEG. The cleared lysate was subsequently sheared and alkaline denatured (pH 12,3) for 10 min in order to eliminate the bulk of chromosomal DNA. Partial renaturation at pH 8,3 followed and protein extraction was undertaken with half the volume of TES saturated phenol. We proceeded to shake out phenol residuals with chloroform/isoamyl alcohol and percipitate the DNA in the aquous solution with twice the volume of ethanol. Centrifugation (100,000 g, -10°C and 45 min) followed and the DNA recollected in 150 µl of TES-buffer, portioned in 20 µl samples and frozen at -20°C.

4.4.1 Electrophoretic investigation of their plasmid DNA content

Electrophoretic investigation of the DNA samples was carried out in the vertical slab gel apparatus. Every sample was mixed with the tracing dye (0,25 vol) and the run was commenced with 120V/ 35 mA/10 min till the dye penetrated the gel. We continued with 22 hrs at 20 V/4 mA. The gel was subsequently put in an EB solution for 3 hrs. The pictureswere shot using 254 nm UV light and a polaroid type camera.
The migration of the different DNA samples, as seen in figure 19, points out, that the mating experiment resulted in the creation of one transconjugant: pJK3 constitutes now a part of the genome of S. faecium M16, herein named S. faecium M16/3. This one derivative is, in comparison with all the other S. faecium M16 derivatives, the one one which did not cease to produce bacteriocin after 40 generations.
The S. faecium M16 derivatives that gained for one generation, at least, the ability to produce bacteriocin, showed, in the migration pattern of their DNA (carried out in a separate electrophoresis run),

only the band representing pM16. This observation provides additional evidence supporting the fact that bacteriocin production is plasmid coded.

Figure 19: Electrophoretic migration pattern of the DNA isolated from (left to right) S. faecium M16/3, S. faecium M16 and S. faecium 3-0. Upper band = pM16, the bright strip starting approx. at the center of each migration track consists of the sheared chromosomal DNA. The lower band consists of pJK3

The strains involved remained also susceptible towards the bacteriocin produced by S. faecium 3-0. S. faecium M16/3 though, be came resistant to this bacteriocin, a confirmation of the fact that immunity has been cotransferred with bacteriocinogenity, i.e. immunity consists an allel of the same gen, as described by Jacob et al, (1975), Halwell and Sherratt (1976) and Inselburg and Johns (1975).

4.4.2 Cleavage procedure of the mating partners and the transconjugant with Eco RI

In the framework of this thesis I made use of the restriction endonuclease as an additional mode of proof for the formation of transconjugants.

The DNA restriction enzymes are endonucleases, which recognize and subsequently cleave specific nucleotide sequences in double stranded DNA. They constitute a part of a restriction and modification system aimed at protecting the bacterial cell from invasive, foreign DNA while the cell DNA is protected by modification mechanisms (i.e. methylation). Not all of the restric - tion endonucleases known today cleave the DNA at a specific nucleotide sequence; this enzyme group is therefore being devided into two classes.

In the following experiment an enzyme belonging to class II, i.e. to the enzymes which cut the DNA at specific base sequences only, was employed. The enzymes belonging to this group have a MW of 20,000 - 100,000 and need Mg^{++} ions as cofactors. The nomenclature of the restriction endonucleases is based on the initial of the microorganism and a number (in roman letters) of the enzyme isolated from it. Eco RI, the enzyme isolated by us, recognizes the sequence G/AATTC.

In comparing the Eco RI digested S. faecium 3-0 and S. faecium M16/3 plasmids, we could draw conclusions concerning the homology of the DNA bands.

For the purpose of determining the MW of the linear fragments resulting from such a cleavage procedure, a hydrolysate of Lambda phage DNA pretreated with the restriction endonuclease Hind III (cleavage site: A/AGCTT) was employed.

Ethanol percipitated plasmid DNA which were planned to be treated by Eco RI were always resuspended in the buffer solution in which cleavage subsequently took place. (0,1 M Tris-HCl, 50 mM NaCl and 10 mM $MgCl_2$, pH 7,5).

25 µl of plasmid DNA isolated from S. faecium 3-0, S. faecium M16 and S. faecium M16/3 were eached mixed with 3 µl of a 1:4 dilution of Eco RI (total dilution factor: 1:24) in a microtiter plate. The plate was subsequently placed in a waterbath and shaked gently at 37°C for 45 min. to allow for optimal digestion. Termination of the cleavage procedure was accomplished by adding 10 ul of a "stopper" solution consisting of 7 mM urea, 0,1 M EDTA, 50% sucrose and 0,1% brome phenol blue. The ingredients point out that this solution fulfilled at the same time the role of a migration marker in the subsequently formed electrophoresis.

The Lambda DNA - length standard was dissolved in distilled H_2O to form a concentration of 1 µg/ml. 4 µl hereof were mixed with 6 µl of the Eco RI stopper solution (see above) and devided into two portions which were applied onto the extreme left and extreme right slots of the gel.
Electrophoresis was carried out by employing Seakam agarose dissolved in 89 mM Tris-base, 2,5 mM Na_2EDTA and 8,9 mM boric acid. It was poured at a temperature of 90-95°C between the vertically fixed glass plates in order to eliminate bubbles easily.

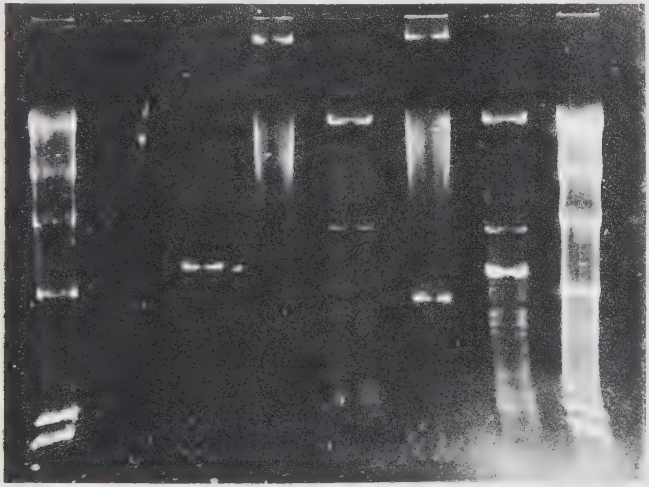

Figure 20: Electrophoretic migration pattern of the following DNA samples (left to right): Hind III digested Lambda phage DNA, S. faecium 3-0 plasmid, S. faecium 3-0 plasmid, Eco RI cleaved, S. faecium M16, S. faecium M16 Eco RI cleaved, S. faecium M16/3, S. faecium M16/3 Eco RI digested and again Hind III predigested Lambda phage DNA. For further details see text.

25 µl of plasmid DNA control samples (not pretreated with EcoRI) were mixed with 10 µl of the dye solution and applied onto the gel. The run was started with 15 min at 120 V/25 mA and continued for

22 hrs at 20 V/4 mA.
The apparatus was cooled down using a constant flow of tap water along the glass plates. After staining the gel with EB it was photographed with the polaroid type camera, exposing the negative for 8 seconds.
As observed in figure 20, DNA cleavage of the different plasmid DNA samples was total; no traces of uncleaved DNA is detectable. The S. faecium 3-0 plasmid proves to have one cleavage site for Eco RI. This is supported by the finding that only one band is observed. This band migrates slower then the CCC form due to the fact that it posseses linear DNA. The plasmid isolated from S. faecium M16 as viewed in the 4th slot from the left, proves to have two cleavage sites: in the following slot we can observe two bands. The diffuse zone of fluorescens seen underneath the pM16 band consists of linear DNA of chromosomal origin. These debris are also digested by Eco RI resulting in numerous, partially diffuse bands, which are statistically distributed along the migration track. The two bands representing the split pM16 plasmid stand out clearly against this background. The same diffuse bands can be observed in the migration track of DNA isolated from S. faecium M16/3. This very track shows a migration pattern which clearly illustrates the homology of size of the transferred plasmid (originating from S. faecium 3-0). The Eco RI cleavage of both plasmids (as shown in the next to the last slot, from left to right) provides us with additional proof as to the identity of both to the "original" pJK3 and pM16. The higher pM16 bands produces two bands, the lower only one band.

4.3.4 Molecular weight determination of the DNA debris following Eco RI digestion

For the purpose of determining the MW of the linear fragments we employed predigested Lambda phage DNA. The migration distance of the linear fragments was measured in mm and drawn as a function of their known MW. (see figure 21). We observe the following fragments from top to bottom of the gel: 1st: 14,63 Mdal 2nd: 6,13 Mdal. 3rd: 4,07 Mdal, 4th: 2,83 Mdal 5th: 1,45 Mdal and 6th: 1,26 Mdal. (data from Boehringer Mannheim). In correlation with the findings

of Helling et al. (1974) we see that the bands consisting of DNA with a MW lower then 2 Mdal are diffuse rather than sharp. The Lambda Phage DNA is "contaminated" with DNA debris of different sizes which is brought out by the relatively stron background fluorescens under UV illumination (due to EB bound to it).
As we see in figure 21, the semi logarithmic plotting of the MW against the distance of migration is linear in the range of 1,5-6 Mdal. DNA fragments which are heavier move quicker within the duration of the run, lighter fragments move too slowly so as to fit on a linear plot.

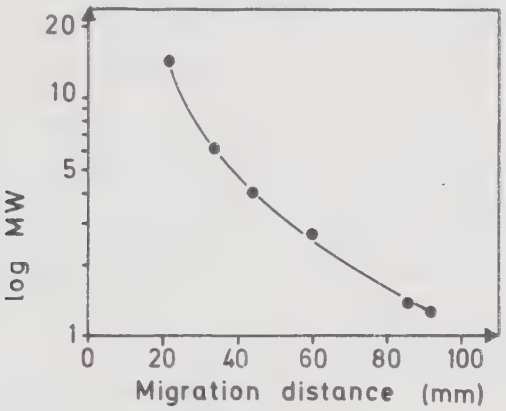

Figure 21: relative electrophoretic mobilities of linear, double stranded DNA in 1,0% Seakam agarose gel.
Distance of migration was plotted against the log of molecular weight.

T e distance of migration of the linear DNA (of the Eco RI digested plasmids) was measured in mm and the equivalent MW determined with the aid of the reference curve. For the linear form of the S. faecium 3-0 plasmid a migration distance of 54 mm from the starting point was measured; this distance corresponds to a MW of 3.25 Mdal (compared to 3,5 Mdal measured with the aid of the electron microscope). The first band of the S. faecium M16 plasmid was measured to have migrated along a distance of 22 mm, corresponding to a MW of 14,2 Mdal. For the second band 44 mm were measured, equivalent to a MW of 4,5 Mdal. Both sum up to a total of 18,7 Mdal.

The electron microscopic length determination showed 19,1 Mdal for this plasmid.
As observed, this method produces satisfectory results. The discrepancy between the electron microscopic length determination and the estimated MW with the aid of the electrophoresis system is significant only in the case of the S. faecium M16 plasmid. The longer linear piece which resulted from its Eco RI cleavage laid outside the zone in which linearity between MW and migration distance is given.

4.5. Characterization of the biological traits cotransferred with the S. faecium 3-0 plasmid

4.5.1 Comparative studies of the bacteriocins produced

In order to provide additional evidence for the identity of the bacteriocin produced by S. faecium M16/3 with the one produced by S. faecium 3-0 the following tests were performed: inhibition spectrum, temperature stability, susceptibility towards proteolytic enzymes, pH stability and possible hemolytic features.

4.5.1.1 Spectrum of inhibition

Donors / Indicators	S. faecium 3-0	S. faecium M16	S. faecium M16/3
S. faecium 160	10 mm	-	16 mm
S. faecium 38	13 mm	-	20 mm
S. faecium 59	14 mm	-	14 mm
S. faecium 202	12 mm	-	14 mm
S. faecium 158	16 mm	-	24 mm
S. faecalis 184	8 mm	-	15 mm
S. faecalis 214	10 mm	-	12 mm
S. faecalis var liquefaciens 151	-	-	-

Table 7: Comparative testing of the bacteriocin inhibition spectrum of the mating partners and the transconjugant. Numbers indicate inhibition zone diameter. "-" = no bacterio

All three donor strains were inoculated (toothpick) in TPB agar dishes divided into three triangles. Incubation followed o.n. at 37°C. At the same time, an o.n. culture in TPB of each one of the indicator strains was prepared. On the following day all donor colonies were killed over chloroform vapor and subsequently overlayed with 3,0 ml of TPB soft agar containing 0,1 ml of the indicator. Evaluation was carried out after an additional 6 hrs of incubation.

As the results presented in table 7 show, the bacteriocin inhibition spectrum of plasmid donor and recipient is the same. S. faecium M16 remained bacteriocin negative. Differences in the size of inhibition zone diameter are due to the different intensity of growth (and therefore bacteriocin production capacity) observed between both strains.

4.5.1.2 Temperature stability

Both S. faecium 3-0 and S. faecium M16/3 were cultivated in 5,0 ml TPB until $\Delta E_{550\ nm} = 0,9$. The cells were subsequently pelleted (15 min 4,000 g) and the supernatant placed in a water bath at 80°C for 15 min. After reaching room temperature it was serially diluted (1:2...1:128). At the same time 0,1 ml of a S. faecium strain 158 culture was resuspended in 3,0 ml TPB soft-agar and poured over TPB agar. 10 µl of each one of the supernatant's concentrations were pipetted onto the solid surface. An o.n. incubation at 37°C followed and the evaluation showed clear zones of inhibition in the indicator lawn up to a titer of 1:256 (both strains). The same test was repeated after havong autoclaved the bacteriocin containing supernatant. This step, however, resulted in total loss of bacteriocin activity.

4.5.1.3 Susceptibility towards proteolytic enzymes

In an effort to classify the bacteriocins concerned, the testing of their digestibility by proteolytic enzymes is a standard method. The supernatant of each S. faecium 3-0 and M16/3 cultures harvested at the end of the logarithmic phase of growth was heated for 15 min at 80°C in order to kill vegetative cells. We proceeded in dividing it into 1,0 ml portions to which 1 mg of the following enzymes was

added: pepsin, trypsin, chemotrypsinogene and pronase. After 30 min of incubation at 37°C the solutions were abruptly frozen (the tubes were dipped into -20°C cold ethanol) in order to stop the enzymatic activity. Controls consisting only of the enzymes enzymes or of the bacteriocin were tested at the same time. Testing bacteriocin activity followed the scheme described under 4.5.1.2.

The evaluation of the control plates showed that the temperature schock did not significantly hamper the bacteriocingenic activity presented in the (not pretreated) supernatant. At a titer of 1:64 a clear zone of inhibition in the indicator strain lawn was observed. It seems that the enzymes applied (no bacteriocin present) were in no way a source of inhibition to the growth of the indicator strain S. faecium 158. It became subsequently apparent, that all the enzymes applied in this assay managed to totally destroy the bacteriocin produced by both strains. No inhibition of growth in the lawn of the indicator strain could be observed.

4.5.1.4 pH stability of the bacteriocins

Two 200 ml cultures of S. faecium 3-0 and S. faecium M16/3 were incubated till $\Delta E_{550nm} = 0.9$. We proceeded to pellet the cells and to heat the supernatant for 15 min at 80°C. After room temperature was reached, the pH of both was measured and found to be 6,13 (S. faecium 3-0) and 6,3 (S. faecium M16/3). We continued in dividing the supernatant to 15 ml portions and adjusted their pH either with NaOH (3M) or $HClO_4$ (11,6 M)from 1 to 13. This adjustment was followed by serially diluting each pH sample (1:2...1:128) and spotting 10 µl of every concentration on TPB agar. The agar had been covered beforehead with 0,1 ml of an o.n. culture of S. faecium 158 in soft agar.

The results presented in figure 22 point out, that the bacteriocin retained in both cases its capacity over a very broad range. where as total loss of bacteriocinogenity occures at a pH of 12,0 the bacteriocin is still active at a pH of 1,0 despite a dilution of 1:64. The differences in the activity of both bacteriocins is of minor significans; it occured only with pH 2,0 and 3,0 where the S. faecium 3-0 bacteriocin seems to be stable (titer: 1:128) then

S. faecium M16/3 (1:64). The pH optimum of both bacteriocins lies between 4-6 with a titer maximum of 1:128. This observation points out that the bacteriocins are both positively charged and their identity is thereby established beyond doubt.

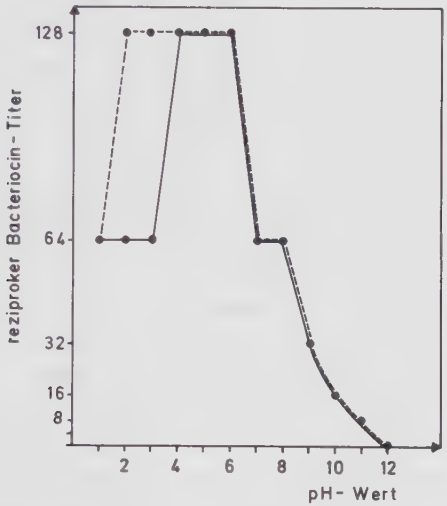

Figure 22: Reciproce bacteriocin titer present in the supernatant of S. faecium 3-0 (- - -) and S. faecium M16/3 (——) as function of its pH.

4.5.1.5 Filterability

A maximum of 20 ml culture supernanatant could be filtered through a Millipore membrane filter with a pore size of 0,45 u. The sterile filtrates of both strains were checked for bacteriocinogenic activity employing S. faecium 158 as indicator. It turned out that a supernatant dilution of 1:64 (compared to 1:128 under normal conditions) still resulted in a zone of inhibition in the lawn of the indicator strain. It is therefore clear, that a certain amount of bacteriocin remained attached to the filter. With this in mind we proceeded to vortex the filters of both strains, each in 1,0 ml TPB, and to determine the minimal inhibition concentration, by spotting 10 ul of the dilutions on indicator strain lawn (TPB agar)

After an o.n. incubation, a titer of 1:256 was observed, at which a clear zone of inhibition was still visible. This value is double as high as under normal conditions.

4.5.1.6 Bacteriocin = hemolysin ?

All the bacteriocin determining plasmids which were transferred within group D Streptococci were at the same time hemolysin producers. (Brock and Davie, 1963; Van Embden et al, 1977 among the many others). As a matter of fact, the authors of the publications concerned could not exclude the possible identity between the bacteriocin and the hemolysin.
For the purpose of determining on which blood type S faecium 3-0 produces hemolysin we employed human, sheep and horse blood. Defibrination of the blood was carried out with glass beads which were shaken vigorously with the blood immediately after puncturing was completed. The blood (5,0 ml) was added to the autoclaved agar base medium (95,0 ml) which was cooled down to 45°C (see material and methods for details). The strains were then streaked on the agar and incubated o.n. The results arrived at point out, that neither S. faecium 3-0 nor S. faecium M16/3 possess the ability to lyse erythrocytes of the blood spplied. The only feature observed was a slight alpha hemolysis on sheep blood produced by S. faecium 3. The remaining two strains built pale white colonies on this medium. We conclude herefrom that the bacteriocin is not correlated with a hemolysin in the system studied by us.

4.5.2 Characterization of the immunity gained by S. faecium M16/3 towards the bacteriocin produced by S. faecium 3-0

As observed in previous experiments, S. faecium M16 proved sensitive towards the bacteriocin produced by S. faecium 3-0.
One change that occured as a result of the mating procedure concerned the immunity gained by the recipient strain towards the bacteriocin. Co-transfer of bacteriocinogenity with immunity has often been described before (Tomura et al, 1978, Basinger and Jackson 1968, Jacob et al 1975). As a confirmation of our expectations we could show, that the bacteriocin produced by S. faecium M16/3 in-

hibits the growth of S. faecium M16. (till a dilution of 1:128). Concerning the immunity, it could often be observed, that the undiluted bacteriocin of S. faecium 3-0 caused a turbid zone of inhibition on an S. faecium M16/3 lawn. (employing the test in which 10 µl of every dilution step were applied on the indicator strain lawn).

In order to clarify whether or not S. faecium 3-0 produced two different bacteriocins of which only one was transferred to S. faecium M16/3, we picked an S. faecium M16 colony, which proved to be resistant to the S. faecium M16/3 bacteriocin (isolated from the inhibition zone as pin-point colony). This mutant colony was cultivated o.n. in TPB broth. At the same time S. faecium strain 3-0 was inoculated in the center of a TPB agar dish. The growing CFU was killed on the following morning with a drop of chloroform. The plate was subsequently overlayed with 3,0 ml of softagar supplemented with 0,1 ml of the S. faecium M16 mutant (6 hr culture). Evaluation carried out 6 hrs later showed that the mutant retained its resistance also towards the S. faecium 3-0 bacteriocin. These results point out that S. faecium 3-0 produces only one bacteriocin because if it had produced two different ones, the resistant S. faecium M16 mutant would have had to prove susceptible to the one which was not transferred.

After having clarified this point, we proceeded to determined the range of the immunity gained by S. faecium M16/3. In figure 24 (right half) we observe a zone of inhibition caused by S. faecium 3-0 in a S. faecium M16 lawn at a dilution of 1:64. The same dilution employing S. faecium M16/3 as donor strain is seen in figure 25 (right half). This dilution is still sufficient to inhibit the same strain prior to its mating with S. faecium 3-0.

A few tests carried out on the same basis as above and employing S. faecium 3-0 as donor with S. faecium M16/3 as indicator, showed that from time to time concentrated supernatant derived from S. faecium 3-0 inhibited the growth of S. faecium M16/3. Continuing experiments on the immunity transfer were carried out as will be shown in the following. (see figures 23-25).

The tests were executed after having killed vegetative cells by heating the supernatant for 10 min at 80°C and diluting it to 1:256. After the agar containing the indicator strain stiffend, the petri dishes were spotted with 10 µl of each dilution.

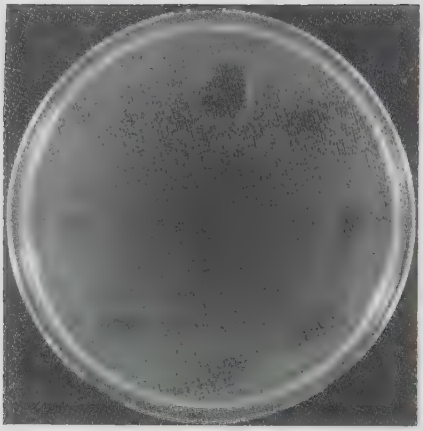

Figure 23: Bacteriocin test employing S. faecium 3-0 as donor and S. faecium M16/3 as indicator

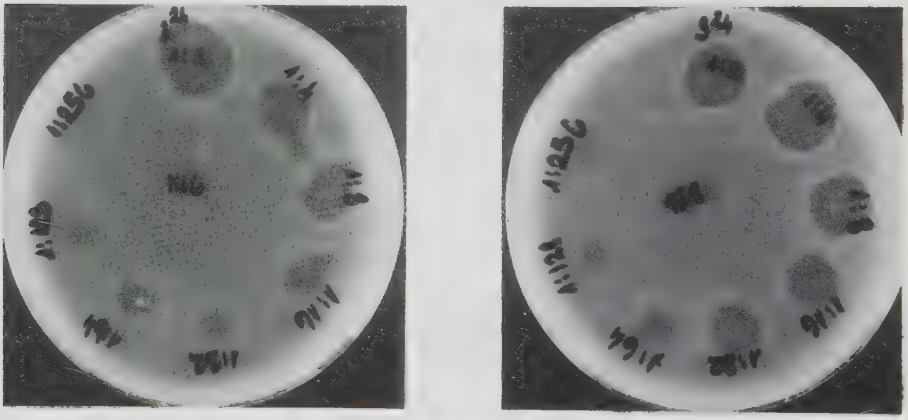

Figure 24: Bacteriocin titer determination: (left to right) 1. donor S. faecium 3-0 indicator S. faecium 158. 2. donor is identical, indicator is S. faecium M16. The numbers give the dilution of which 10 ul were spotted on the indicator lawn.

The resistant colonies observed in the inhibition zones are those of the bacteriocin donor strain; as we see, the higher the bacteriocin dilution is, the lower the number of these colonies.

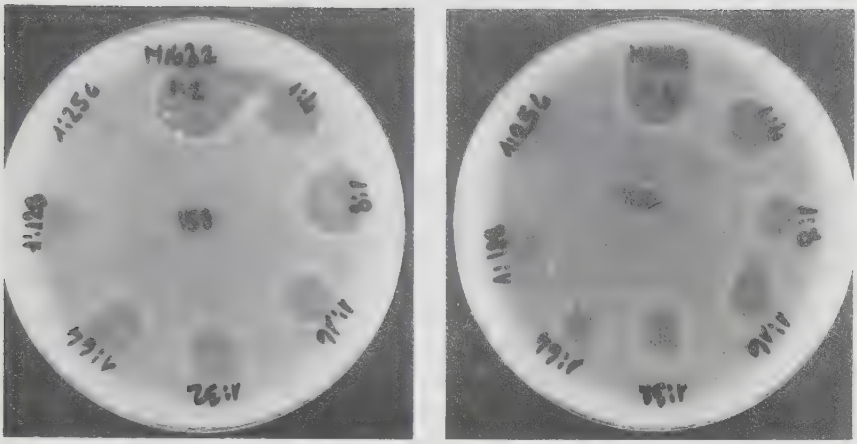

Figure 25: Same as in figure 24, only this time the donor, in both cases, is S. faecium M16/3 and the indicator (left) S. faecium 158 and (right) S. faecium M16.

4.6. Attempt to clarify the pJK3 transfer to S. faecium M16

Due to the fact that the conjugal transfer of the S. faecium 3-0 plasmid to S. faecium M16 did not follow any of the models known to us, i.e. was not promoted by mobilization through a bigger plasmid (practically impossible that a 3,5 Mdal plasmid would be in a position to code for bacteriocinogenity, immunity towards the same, its own replication and also carry the genes responsible for transfer) we decided to examine the possibility, that the S. faecium M16 plasmid was transferred into S. faecium 3-0 during the mating. This speculating that a two-way-transfer of the latter plasmid took place in the 18 hrs of conjugation. It could be that this plasmid acted as mobilizing agent.
S. faecium 3-1 and S. faecium M16 were inoculated into 5,0 ml of M9 medium and incubated o.n. The mating mixture prepared on the next morning consisted of 0,1 ml S. faecium 3-1 culture, 0,4 ml of the S. faecium M16 culture and 4,5 ml M9 medium. After o.n. incubation the filter through which this mixture was forced, was resuspended in 1,0 ml broth and 0,1 ml portions here

from were streaked on rifampicin, fusidic acid (each 20 µg/ml) and erythromycin (10 ug/ml) supplemented agar. Controls of each one of the mating partners were also prepared. The evaluation, carried out after 48 hrs of incubation (to allow phenotypic expression) produced no erythromycin resistant (and therefore pM16 harboring) S. faecium 3-1 transconjugants. The controls remained negative as well. The results represented here were reproducible during the three times this experiment was repeated. In the following, we repeated this experiment employing the transconjugant S. faecium strain M16/3 as donor and S. faecium 3-1 as recipient. This was undertaken, in order to determine whether or not the presence of pJK3 in the S. faecium M16 strain had any influence on pM16 transferability. Here we could use one equal amount of donor and recipientin the mating mixture because of the immunity S. faecium M16/3 gained towards the S. faecium 3-1 bacteriocin. In this case as well we failed to isolate S. faecium 3-1 derivatives which gained resistance towards erythromycin.
These findings point out, that pM16 cannot be counted upon as mobilizing agent for pJK3 in the manner assumed in the above hypothesis, furthermore, its self-transferability is put in question.

5. Evidence indicating plasmid coded sucrose utilization in S. faecium 25-0

5.1. Transfer of the S. faecium 25-1 plasmid into S. faecium M16

In proceeding our investigations as to the transferability of the S. faecium M16 plasmid, we prepared a mating mixture consisting of this strain and S. faecium 25-1 as recipeint. We were able to isolate only reciprocal transconjugants, i.e. S. faecium M16 derivatives, which harbor S. faecium 25-1 plasmids (as will be proved later on). The mating was performed as follows: 0,25 ml of an o.n. culture of each one of these strains were mixed with 4,5 ml M9 medium and forced through a filter. The filter was incubated o.n. after which it was resuspended in 1,0 ml broth. Selection for transconjugants was performed on rifampicin, fusidic acid and erythromycin supplemented agar. Controls consisting of S. faecium M16 (streaked on rifampicin and fusidic acid supplemented agar) and of

S. faecium 25-1 (streaked on erythromycin supplemented agar) were also set. The evaluation, carried out after 48 hrs of incubation at 37°C produced three CFU reistant to all three antibiotics applied. The controls were all negative. The three isolates were purified on blood agar: we observed that the colonies were slightly bigger then those of S. faecium 25-1 and much more similar to thos S. faecium M16 produces.

5.1.1 Transconjugants analysis: carbohydrate utilization and antibiotics resistance spectra

The further phenotypic characterization of the above mating products dealt with sucrose utilization. (S. faecium M16 is sucrose negative). S. faecium 25-1, S. faecium M16 and the three isolates from the previous experiment were inoculated into CTA medium supplemented with 1,0% sucrose and bromephenol purple as pH indicator. As expected, following 24 hrs of incubation S. faecium 25-1 and the isolates proved sucrose positive where as S. faecium M16 did not. At last a bacteriocin test was carried out. All strains involved were employed as donors. Each was inoculated (toothpick) on TPB agar, incubated o.n. killed subsequently with chloroform and overlayed with 3,0 ml soft agar supplemented with S. faecium 158 as indicator.
The evaluation carried out 6 hrs later proved that none of the strains but S. faecium 25-1 produced a zone of inhibition in the S. faecium 158 lawn.
In the following all strains were streaked on tetracycline (10 µg/ml) supplemented TPB agar; S. faecium 25-1 was the only one susceptible. The three on rifampicin, fusidic acid and erythromycin isolated derivatives were resistant towards this antibiotic. (Although not selected for this feature).
As determined elsewhere, raffinose utilization constituted another differentiation marker between S. faecium 25-0 (raffinose +) and S. faecium M16 (raffinose -). Our experiments proved that the derivatives did not possess the ability to utilize this sugar as a carbohydrate source.
These facts and the morphological similarity of the transconjugants with S. faecium M16 (on blood agar plates) were sufficient proof

for the isolates to be classified as transconjugants of S. faecium strain M16. They gained the ability to utilize sucrose and to resist the action of rifampicin and fusidic acid.

5.1.2 Transconjugant's analysis: determination of MIC towards rifampicin and fusidic acid

Determination of the minimal inhibition concentration of the S. faecium M16 derivatives towards the acquired antibiotic resistance were carried out in tryptose phosphate broth.
Rifampicin and fusidic acid were dissolved in methanol which was poured into prewarmed (39°C) broth and then immediately vaporized.

strain designation	minimal inhibition concentration	
	rifampicin	fusidic acid
S. faecium 25-1	2 mg/ml	1,125 µg/ml
S. faecium M16	31,25 µg/ml	3,9 µg/ml
S. faecium M16/25-1	2 mg/ml*	2 mg/ml
S. faecium M16/25-2	2 mg/ml*	2 mg/ml
S. faecium M16/25-3	2 mg/ml	2 mg/ml

Table 8: MIC of rifampicin and fusidic acid towards the donor, recipient, and recipient strain's derivatives as determined in TPB. (*solubility product of rifampicin lies slightly above 2 mg/ml).

Each of the strains tested was cultivated o.n. in TPB and diluted subsequently 1:100 in fresh broth. The tubes containing the antibiotic concentrations were each inoculated with 0,01 ml here of. Evaluation was carried out as soon as the control tube (containing no antibiotics) showed significant growth.

The results point out that the resistance shown by all the recipient strain's derivatives lies within the same level for each of the antibiotics tested. The resistance levels acquired by them towards rifampicin, was the same as with the donor strain S. faecium 25-1. Their resistance towards fusidic acid however, was 1,6 higher then the donor's resistance level. Repeated experiments

and reports by Kühnen (1980) however, indicate that S. faecium M16 tends to spontaneously mutate in high frequencies to rifampicin and fusidic acid resistance.

5.1.3 Transconjugant's analysis: electrophoretic investigation of their plasmid DNA content

The selection of S. faecium M16 derivatives which are capable of utilizing sucrose arose our interest as to a possible correlation between this feature and the acquisition of plasmid DNA from S. faecium 25-1.
S. faecium M16 (negative control), S. faecium 25-1 and the three S. faecium M16/25 derivatives were each cultivated o.n. in M9 medium. 0,1 ml of each of these cultures was inoculated into 75 ml M9 medium (which was supplemented with 10 ug/ml erythromycin as selection pressure for pM16 in the strains harboring it). After reaching an O.D. of 0,85 (550 nm) the cells were harvested , resuspended in hypertonic sucrose solution and lysed with lysozyme, EDTA and a mixture of sarkosyl/PEG, and kept o.n. at -20°C.
We proceeded the following morning to shear the laysate by employing a gaugeless syringe. Alkaline denaturation and protein extraction with phenol followed the scheme as described under material and methods.
The DNA was percipitated with ethanol and 30 ul of the pooled percipitates were applied for electrophoretic investigations.
Figure 26 shows us the migration pattern of the plasmids harbored by the donor- and recipient strains as well as the transconjugants. The outstanding feature of the transconjugants is their homologous plasmid DNA content. All three of them show, next to the pM16 plasmid band, two new plasmid bands usually harbored by S. faecium strain 25-0. The upper band is identical with the 4,7 Mdal, the lower with the 2,4 Mdal plasmid. No sign of transfer of the 13 Mdal plasmid is observed. The band appearing above this very plasmid (S. faecium 25-1, second slot from left) represents its OC form.

It appears that the ability to utilize sucrose does correlate with the transfer of plasmid DNA. Moreover, there seems to be a correlation between the "mini" and "midi" plasmids derived from S. faecium 25-1: both were transferred "en bloc" to all three trans-

conjugants, which were, for their part, isolated independently of one another.

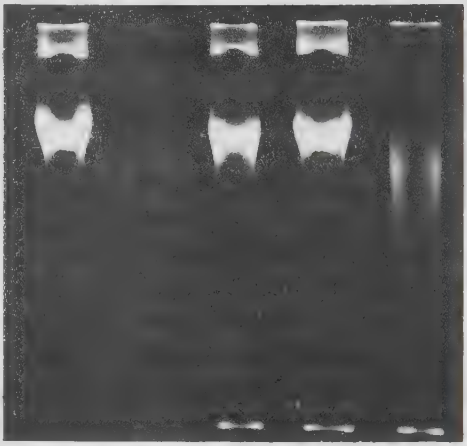

Figure 26: Agarose gel electrophoresis of (left to right) S. faecium M16, S. faecium 25-1 and the S. faecium M16/25-1, M16/25-2 as well as M16/25-3 transconjugants. Migration was from top (anode) and carried out for 22 hrs at 20 V/4 mA.

The upper band of the first slot represents pM16 plasmid DNA. The upper band of the second slot represents the OC form of S. faecium 25-1 "maxi" plasmid, below it, the CCC form of the same plasmid is visible. The two lower bands are the "midi" followed by the "mini" plasmid DNA derived from the same strain. The following three slots show uniformly the pM16 followed by the "midi" and "mini" plasmid bands transferred from S. faecium 25-1. The thicker band observed in all slots at the upper half of the migration track consists of chromosomal DNA debris.

5.1.3.1 Comparative Eco RI digestion of the mating partners and the transconjugants

For the purpose of determining the cleavage sites on the S. faecium 25-1 plasmid DNA as well as provide additional proof for the formation of transconjugants, an electrophoretic run of the Eco RI digested plasmids was performed.

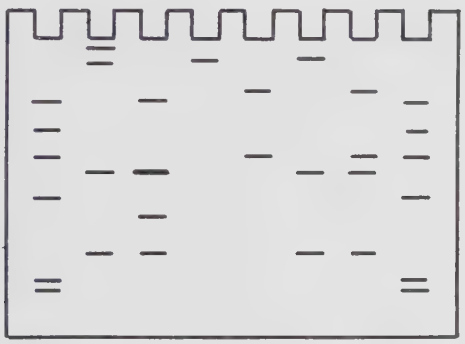

Figure 27: DNA bands as separated in agarose gel electrophoresis. The run was performed for 22 hrs at 20 V/ 4 mA after which migration pattern was made visible in staining the gel with ethidium bromide.
Samples are (left to right): Lambda phage DNA length standard, S. faecium 25-1, S. faecium 25-1 plasmids Eco RI predigested, S. faecium M16, S. faecium M16 digested plasmid, S. faecium M16/25-1, S. faecium M16/25-1 Eco RI cleaved and at last another sample of the Lambda phage DNA length standard.

The content of the samples was the following for all strains examined in this test:

20 µl plasmid DNA
10 µl dilution buffer
10 µl reaction buffer
2,5 µl Eco RI enzyme solution

(see material and methods for buffer formula). The ingredients were

mixed thoroughly and incubated for 45 min in a water bath. The reaction was brought to a halt by adding 15 ul of a stopper solution to it.

Figure 27 is a presentation of the migration pattern of the non- and predigested plasmids including Lambda phage hydrolysate as MW marker. The upper band in the slot containing the S. faecium 25-1 plasmids consists of the OC form of the "maxi" plasmid. Lower then the chromosomal band we observe in the same migration track the "midi" and "mini" plasmid bands. As we see in the following slot, that "midi" and "mini" plasmids do not possess nucleotide sequences which can be cleaved by Eco RI. The bands representing these plasmids remain unchanged after enzymatic treatment. The "maxi" plasmid with its MW of 13 Mdal proves, however, to possess two cleavage sites. This plasmid splits into two linear pieces (notice that its OC band disappears as well). The MW of these linear debris (calculated as function of their migration distance in relation to the migration of the Lambda phage MW standard) is 10 Mdal for the upper, and 2,6 Mdal for the lower band. (which is located between the non-digested "midi" and "mini" plasmid bands). These values add up to a total of 12,6 Mdal - a value which lays very close to the one determined electron microscopically. The S. faecium M16 plasmid proves to have two cleavage sites for Eco RI (as already described in connection with figure 24).

The second before last slot shows the migration pattern of the plasmids isolated from the S. faecium M16/25-1 transconjugant. (analysed in fig. 26). The following slot shows these plasmids following their Eco RI digestion. The only plasmid which cleaved is pM16 with the same migration pattern seen at the S. faecium M16 DNA slot. The "midi" and "mini" plasmids transferred from S. faecium 25-1 have no Eco RI cleavage sites - this is an additional support for their origin.

5.2. Sucrose utilization by S. faecium 25-0 and S. faecium M16/25-1: quantitative determination

The rough determination of sucrose utilization as function of acid production (change of indicator color = sugar utilization) does not constitute an objective standard but serves rather as an indication. In order to study this point a colorimetric method of sucrose uptake (described by Bergmeier and Berendt, 1974) was performed.

S. faecium s5-1, S. faecium M16/25-1 as well as S. faecium M16 were cultivated o.n. in peptone water supplemented with 0,02 M sucrose. S. faecium M16 was excluded from further investigations because it could not utilize sucrose as carbon source; the O.D. of its o.n. culture never exceeded 0,09 - 0,15 at 550 nm.
1,0 ml of the first two cultures were pipetted into the main culture (100 ml) of which 5,0 ml were immediately taken for quantitative sucrose determination. Cultivation was carried out in a water bath at 37°C under slight agitation. Samples were taken at different stages of growth (see figure 28 and 29), their turbidity measured and the cells subsequently pelleted. Sucrose determination was carried out (employing supernatant). For further details see material and methods.
Observing figure 28 we find out, that at t=0 the colorimetric determination of sucrose concentration proves to correlate well with the quantity of the sucrose supplemented to the medium. The relation between sucrose uptake and growth is (inverse-) linear during the entire cultivation.
Logarithmic phase of growth began at t=3 hrs and a stationary phase was reached after a total of 9 hrs. This phase was rather short (1 hr) and its end was marked by maximum sucrose utilization, i.e., half of the total quantity (0,01 M) were metabolized. (at an O.D. of 0,72, 550 nm). In the following experiments cultures were harvested at this very stage for ß-fructosidase assay. The stationary phase was followed by a phase of increased death rate. This was due to cell autolysis (resulting also in the uptake of turbidity) which caused the setting free of sucrose.
In figure 29 we observe that S. faecium 25-1 is a very inefficient

sucrose utilizer. After 19 hrs of growth it metabolizes almost the same amount of sucrose S. faecium M16/25-1 does in half the time (7 mM compared with 10 mM) and this with an O.D. of merely 0,32 (at 550 nm) compared to the O.D. of 0,72. The rather flat logarithmic phase of growth is stretched over a period of 10 hrs; its peak correlates with maximal sucrose utilization.

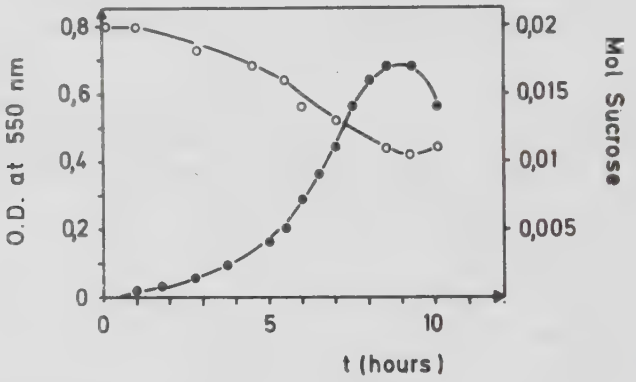

Figure 28: Growth curve of S. faecium M16/25-1 (•) in relation to sucrose uptake (o) in the supernatant

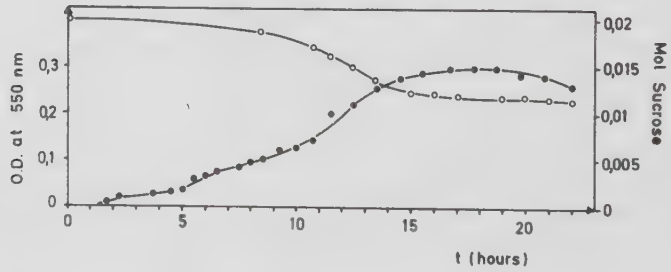

Figure 29: Growth of S. faecium 25-1 (•) and uptake of sucrose concentration (o) in the culture's supernatant.

5.3 Evidence of ß-Fructosidase in crude cell extracts

Having demonstrated sucrose consumption by S. faecium 25-1 and the transconjugant S. faecium M16/25-1, we decided to proceed these investigations with the aim of isolating the enyzme responsible herefor. This could eventually be a ß-Fructosidase. The ß-Fructosidase or ß-Fructofuranosidase hydrolyses the bonds between the fructose carbon and the bridge oxygen (as described for baker's yeast). The process of hydrolysis explains the pH activity curve on sucrose. This curve shows a broad optimum range of pH 4,5-5,5 (Bergmeier and Berendt, 1974). These details constituted the basis of the following investigations

3,2 liter peptone water were supplemented with 0,02 M sucrose were inoculated with S. faecium 25-1, 2 liters with S. faecium M16/25-1 and 5 liters with S. faecium M16. The cultures were harvested when O.D. reached their optimum as described in the last experiment. (see figure 28 and 29). S. faecium M16 was harvested at ΔE_{550nm}= 0,12 (10 hrs of growth). Vell washing with phosphate buffer pH 7 (0,1 M) was followed by the determination of their wet weight: S. faecium 25-1 cells were 1,84 g (resuspended in 5,52 ml phosphate buffer), S. faecium M16 1,2 g (resuspended in 3,6 ml) and S. faecium M16/25-1 2,08 g (resuspended in 6,24 ml). Following cell disruption we pelleted their debris and performed quantitative protein determination in the remaining supernatant (Biuret methid, see material and methods for details).
The crude extract won from S. faecium 25-1 contained 19,5 mg/ml (i.e. a total of 108 mg), S. faecium M16 17,25 mg/ml (a total of 62,1 mg) and S. faecium M16/25-1 27,5 mg/ml, this makes a total of 171 mg protein.
Since the activity of ß-Fructosidase was measured be means of glucose set free (before and after sucrose hydrolysis), we had to determine the amount of glucose contained in the crude cell extract. 250 µl of each of the extracts were boiled to abolish enzymatic activity and 0,1 ml herefrom were used for the test.
The O.D. measured at 340 nm were as follows: S. faecium 25-1 and S. faecium M16: 0,04 (equivalent to 3,0 µg/ml or 16,6 µM) (see material and methods for mode of calculation). S. faecium

strain M16/25-1 0,05 (equivalent to 4,3 µg/ml or 23,8 µM). These results enable us to disregard glucose determinatibn during the ß-Fructosidase assay.
In the following test assay this crude cell extract was employed as ß-Fructosidase source.
The test was carried out with 15 µl of S. faecium 25-1 crude extract (equivalent to 300 µg protein), 20 µl of S. faecium M16 (equivalent to 345 µg protein) and 10 µl of S. faecium M16/25-1 which contained 275 µg protein. The enzymatic reaction partners had to be supplemented with 0,1 M citrate buffer pH 4,6 (0,3 ml) otherwise sucrose would not have been cleaved.

No enzymatic degradation of sucrose could be demosnstrated with the crude cell extract of S. faecium M16. 30 minutes after the test had been started ba adding G-6-P-DH and HK), an O.D. of 0,03 (at 340 nm) was measured. This value was read at E1 and represents reduced NADP in the crude extract as well as in the sample purchased for the performance of this test.
As for the definition of enzyme activity, one enzyme unit is considered as the quantity of enzyme which reduces 1 µM NADP per min. Specific activities are presented as units or milliunits per mg protein.

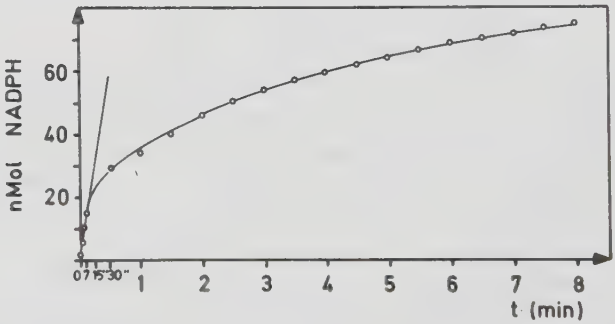

Figure 30: Reduced NADP in relation to the reaction time following sucrose cleavage by ß-Fructosidase present in the crude cell extract of S. faecium 25-1.

Figure 30 provides us with data concerning the amount of built NADPH (in nM) in relation to the reaction's time. The extrapo-

lation of the first three values (which lay on a linear course) enabled us to determine, that 120 nM NADP would have been reduced per minute, providing the reaction would have followed its linear course.

The specific activity of the ß-Fructosidase is calculated as:

$$\frac{0,12\ \mu Mol\ NADPH}{1\ min \cdot 0,3\ mg\ protein} = 0,4 \text{ or } 400 \text{ milli-units/mg protein.}$$

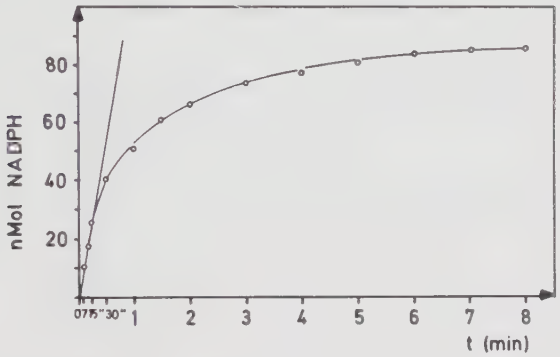

Figure 31: Built NADPH in relation to the reaction time following sucrose cleavage by ß-Fructosidase. Enzyme source was the crude cell extract of S. faecium M16/25-1. Ingredients of the assay were: 15 µl crude cell extract, 2 mM sucrose, 0,1 M citrate buffer pH 4,6 , 0,75 M triethanol amin pH 7,6, 10 mM $MgSO_4 \cdot 7\ H_2O$, 12 mM NADP, 81 mM ATP, 0,6 M $NaHCO_3$, 2 mg/ml HK, 1 mg/ml G-6-P-DH (0,2 ml).

In figure 31 we observe that within the first 30 seconds 54 nM of NADP would have been reduced if the reaction would have followed the linear course observed in the first 12 seconds. Specific activity is therefore calculated to be:

$$\frac{0,108\ \mu Mol\ NADPH}{1\ min \cdot 0,275\ mg\ protein} = 0,39 \text{ or } 390 \text{ milli-units/mg protein.}$$

This value correlates well with the one determined for S. Faecium strain 25-1.

We can state therefore, that the ability to utilize sucrose was obtained by the S. faecium M16/25-1 transconjugant through transfer of capacity to synthesize ß-Fructosidase.

This feature has been co-transferred with the "midi" and "mini" plasmids from S. faecium 25-1. The possibility of its genetical determinants to be located on this DNA cannot be considered accidental.

DISCUSSION

Bacterial plasmids are defined as cytoplasmatic, genetic elements, that are physically independent of the host chromosome. Plasmid are under most conditions non-essential for the primary functions in gene expression of the bacterial cell.
Plasmids were first dicovered in the Enterobacteriaceae but now it seems that they can be found in virtually any bacterial species.
The phenotypic traits coded by plasmids are manifold: antibiotic resistance, antibiotic synthesis, pathogenicity, metabolic functions and bacteriocin production.
In the framework of this thesis it was our aim to investigate the phenotypic traits coded by plasmids harbored by bacteriocin producing strains of S. faecium.
One of the basic requirements herefore was the working out of a DNA enrichment procedure. Such a procedure is involved with the cultivation of reater culture volumes. This fact confronted us with two major factors not counted upon in the direct CsCl-EB-density gradient centrifugation (Meselson et al, 1957) - the extraction of cell protein and that of the bulk chromosomal DNA. Both would otherwise greatly interfere with the density of the gradient formed, disregarding the apperent overlapping of the plasmid band by the chromosomal one.
For the purpose of getting rid of the chromosomal DNA, a step consisting of alkaline denaturation was introduced. At a pH of 12,0 and above, the double stranded DNA separates into its single strands (Bazaral and Helinski, 1968) - the performance of this step followed an intensive shearing of the cleared lysate through which breakage of the DNA to linear fragments resulted.
The re-titration to pH 8,4 results in a partial and incomplete renaturation: the majority of the single strands remains as such and is not ethanol percipitated. The majority of the CCC DNA is not affected by this step because of its compact form.
For deproteinization purposes Marmur (1963) employed phenol and subsequently chloroform/isoamylalcohol (24:1). The phenol employed was saturated with 3% NaCl (total) to maintain ionic

strength of the dissolved DNA. Yagi et al. (1978) and Bazaral and Helinski (1968) report both that neither alkaline denaturation nor phenol extraction caused the destruction of integrity of super coiled DNA.

Chloroform is an excellent protein surface denaturer. Isoamylalcohol was employed because it reduced foaming, aids the phase separation and maintains the stability of the laysers of the centrifuged, deproteinized solution.

Selective increase of plasmid DNA yeald could be clearly demonstrated. A 3,3 fold increase in culture volume resulted in a 5,6 fold higher radioactivity yeald within the plasmid peak (although total radioactivity in the culture was reduced to a third compared to the "standard"culture).

Before puncturing the bottom of the tubes (CsCl-EB-density gradient) we could observe,below the chromosomal band, the plasmid band, which appears only in the enriched DNA preparation. We were able to make this observation without a UV illuminator.

For a more intense plasmid characterization we proceeded our investigation with an electron microscopic plasmid DNA preparation. For the purpose of finding as much CCC-DNA as possible, we prepared the specimen on the same day of CsCl-EB-density gradient evaluation. As marker for length determination we employed plasmid pAD1tc. This plasmid has a MW of 38 Mdal and constitutes a recombination between pAD1 and a tetracycline transposon as executed by Arth Franke (University of Michigan, personal communication). pAD1 itself codes for bacteriocin and hemolysin production and its MW is 35 Mdal.(Tomich et al., 1979). Next to the "mini" plasmid harbored by S. faecium 3-0 we could demonstrate the two tertiery structural forms of the "maxi" plasmid isolated from S. faecium 25-1: OC and CCC. The relaxation phenomenon is due to a single strand nick (Clowes, 1972). Most probably the transitional stadium between the CCC and OC form (fig 5) demosntrates the molecule's configuration following immediately such a nick.

The length determination of the plasmid molecules represents an average of 10 diffeent measurements carried out on photos shot at a constant magnification in one day. All samples to be studied were mixed with the marker plasmid prior to their investigation.

in order to increase objectiveness of the data.
Burgi et al (1963) report, that the zonal centrifugation of mixtures of two labeled DNAs at low concentration in linear sucrose gradients permits accurate measurement of relative sedimentation rate. They found out as well, that the MW of one isotopically labelled DNA can be measured in terms of that of another. This value can be derived from the DNA migration distance as measured in the gradient.
We applied the above relation and compared the so calculated plasmid MW to the ones measured electron microscopically. As plasmid marker we employed pAM 1 which determines tetracycline resistance. This plasmid is one of three harbored by S. faecalis DS5; its MW is 6 Mdal and the sedimentation coefficient of its CCC form is 28S. Thass data were published by Dunny and Clewell (1975). In 1976, Yagi and Clewell transferred pAM 1 to S. faecalis DT11 and this strain was employed as marker source in our experiments. Clowes et al (1972) reported a simple experimental procedure of differentiating between OC and CCC plasmids - the alkaline sucrose gradient (pH 12,0).
Under such conditions, the CCC form becomes even more compact and migrates swifter than the OC form. The latter form splits into single strands due to breakage of hydrogen bonds and migrates as a compact random coil.
In demonstrating the sedimentation profile of the S. faecium 3-0 plasmid DNA, we observe one peak only, which shifts under the same centrifugation conditions (this time in an alkaline gradient) much swifter than in the neutral gradient. We always applied DNA which was isolated on the same day via the CsCl-EB-density gradient because spontaneous conversion from the CCC to the OC form was rather rapid. This process could also be attributed to the fact, that the DNA was marked with radio isotopes. The decomposition (of ^{3}H or ^{14}C) had its mechanic impacts in as much as it caused single strand nicks (Grinsted et al, 1972).
The cosedimentation of pAM 1 with the S. faecium 3-0 plasmid, resulted in a calculated sedimentation coefficient of 22S. This value correlates well (21,8) with the theoretical value figured according to the formula of Bazaral and Helinski (1970).

In the following experiments, the S. faecium 3-0 plasmid was employed as sedimentation marker.

According to the electron microscope investigations, S. faecium 25-0 harbors three plasmids, of which the "mini" and "midi" ones lay within a relatively narrow range of size (2,4 and 4,7 Mdal respectively). The demonstration of the migration profile of the plasmid DNA isolated from this strain shows a rather broad peak at the top of the gradient and a smaller one before it. The same DNA overlayed on alkaline sucrose gradient shows three peaks. For the purpose of analysing these results we cosedimented this DNA with the ^{14}C-marked S. faecium 3-0 plasmid. The number of drops collected per fraction was reduced to 1 in order to stretch the x-axis. We observe that the first broad peak consists actually of two peaks with sedimentation coefficients of 21S and 24S. Both correlate with the values figured for the CCC form of the "mini" and "midi" plasmids. The last band was figured to migrate with a sedimentation coefficient of 38S. This very value is correct for the calculated Sccc form of a 13 Mdal plasmid (the "maxi" plasmid). The migration of these plasmids in alkaline sucrose gradients yealds the same peak indicated already. We could now determine that its sedimentation coefficient is 29S (relative to the neighboring peaks).

Clowes et al (1972) figured a ratio of 1,3 between Sccc/Soc for a 13 Mdal plasmid, this reveals the identity of this band. Similar observations were made by Crawford and Bates (1979), who employed the same DNA isolation procedure and observed next to the CCC band always an OC band with the bigger plasmid (15 Mdal). No OC bands could be demonstrated for the "mini" plasmids they investigated.

Clewell et al (1974) reports that the larger super coiled plasmid molecule (34 Mdal) which he isolated from a S. faecalis strain tends to undergo spontaneous nicking - an observation he could not make with the other smaller plasmid isolated from this strain.

For the purpose of identifying the phenotypic traits coded by the plasmids investigated in this thesis, two principal approaches were applicable: 1. their elimination with the aid of intercalating dyes followed by screening after lost phenotypical pro-

perties (negative evidence) and 2. their transfer into appropriate recipients, which followed by the screening for the features gained by them (positive evidence).

Due to the fact, that plasmid elimination attempts remained fruitless (see also Keness, 1976) we proceeded with transfer attempts of the plasmid DNA.

It appeared until recently, that the transmission of genetic material by conjugation processes was restricted to gram negative bacteria, and occured rarely if at all in gram positive bacteria (Clowes, 1972). Raycroft and Zimmerman (1964) first suggested that the conjugal transfer of genetic material might occur among gram positive bacteria (in explaining intraspecific chloramphenicol transferred resistance in S. faecalis). The actual demonstration of plasmid mediated gene transfer was reported in 1974 by Jacob and Hobbs. Since the latter report, four groups have demonstrated conjugative transfer of plasmids in Enterococci: Clewell et al. (1974), Marder and Kayser (1977), Le Blanc et al (1978) and Gibson et al (1978). Clewell et al. found that plasmid pAMβ1 mediated its own transfer as well as that of the tetracycline-resistance plasmid pAMα1 to other S. faecalis strains. Le Blanc et al. found that pAMβ1, which confers erythromycin and lincomycin resistance, is laso capable of self-transmission. Gibson and coworkers reported that the same plasmid could be intergenerically transferred by means of conjugation from a Lancefield group F Streptococcus to a strain of Streptococcus avium. Both strains served as pAMβ1 donors for three strains of Lactobacillus casei. Marder and Kayser transferred a 26 Mdal plasmid determining chloramphenicol and erythromycin resistance to S. faecalis JH2-2. Based upon these phenomena it could not be outruled, that similar mechanisms of transfer could apply to the strains employed by us.

Small- or "mini" plasmids (MW $<$7 Mdal) are considered to-date to be non-selftransmissible. This observation stems from the fact, that such small genetic elements could code only for a limited number of proteins. Primarily they code for their own replication, and than for a phenotypic trait. Genes coding for plasmid transfer to other microorganisms need much longer DNA segments. Rownd and coworkers (1975) found out that the transfer segment of an R-fac

tor (harbored by a Proteus mirabilis strain) had a MW of 16,5 Mdal. none of the plasmids investigated in this thesis reached this size.

Mini-plasmid transfer mediated by bigger, transferable plasmids, has often been described. This process of "mobilization" was observed by Dunny and Clewell (1975). This team reports on a S. faecalis strain DS-5 which harbors three plasmids: pAMα1, pAMβ1 and pAMγ1 (6, 17 and 34 Mdal respectively). Conjugation of this strain with S. faecalis JH2-2 gives rise to transconjugants carrying pAMα1 (determining tetracycline resistance) and or pAMγ1 (coding for production of hemolysin and two bacteriocins). It was found, that S. faecalis JH2-2 derivatives, carrying both pAMα1 and pAMγ1 or pAMγ1 only, can donate their plasmids to other recipients. On the other hand, derivatives carrying only pAMα1 cannot serve as donors. This clearly indicates the mobilizing features of pAMγ1 towards the nontransmissible pAMα1.

Tally and Snydman (1979) report on reproducible cotransfer of a 20 Mdal plasmid with a 2 Mdal plasmid in Bacteroides fragilis. The smaller plasmid was found to be responsible for clindamycin resistance. Dougan et al (1978) report on the mobilization of the Col E1 plasmid (MW 4,3 Mdal) to be governed by a gene designated "Mob". "Mob"$^-$ is mobilized at a markedly reduced frequency relative to "Mob"$^+$.

Williams et al (1978) report on a morganocinogenic plasmid (MW 3,6 Mdal, Sccc=22), which could be transferred with a mobilizing selftransferable plasmid. Similar observations were made by Milliken and Clowes (1972) who investigated non-selftransferable R-factors which could be transmitted to other cells. This was possible due to the simultaneous presence of a transfer factor "Δ" which is an independent plasmid molecule (MW 60,6 Mdal). Based upon these findings, Oliver et al (1977) described a system aimed at revealing the genetic determinants located on non-selftransferable plasmids. Oliver and her corworkers described a cryptic plasmid pAM539 harbored by S. faecalis ND539. The gene transfer which employed the mobilizing properties of the 34 Mdal pAMγ1 (introduced into this strain beforehand) enabled the construction of isogenic pairs: the plasmid free S. faecalis JH2-2

was used as mediator. It differed only in the presence or the absence of the cryptic plasmid pAM 539. A comparison of pair members (S. faecalis JH2-2 harboring pAMβ1 and its derivative harboring pAMβ1 and pAM539) revealed a consistent positive correlation between the presence of the plasmid and between the bacterial sensitivity to a bacteriocin produced by S. faecium 592.
We decided to make use of this approach employing the self-transmissible plasmid pAD1tc (MW 38 Mdal) coding for hemolysin, the production of bacteriocin and tetracycline resistance. (As determined among others by Tomich et al, 1979). This plasmid has been shown to possess mobilizing capabilities of non-transferable plasmids.
We commenced the series of mating attempts with the selection for recipient's clones that carry chromosomal resistance markers towards the antibiotics rifampicin and fusidic acid. The selection on two antibiotics gave us an additional tool to ensure recipient's reselection and donor's extermination.
The mating experiments were performed employing the filter method. It should be noted in this connection, that mating attempts in liquid media produced either extremely low rates of transconjugants or, what usually happened, none at all. Stuy (1979) made similar observations in mating experiments with Haemophilus influenzae. He brought evidence, that efficiency of conjugation in suspension becomes substantially increased when the latter is poured on the surface of agar and reincubated. Biswas et al (1979) employed the same mating procedure with Neisseria gonorrhoeae. Gibson et al. (1979) reported of similar results. This team reports as well that intergeneric mating experiments are independent of cellular form (cocci or rods).
In as far as hemolysin production as determined by pAD1tc is concerned, we made the observation that this feature is repressed in the earlier stages of growth. The derepression of this characteristic is probably due to the consumption of the growth medium's compounds which leaves the erythrocytes untouched.
Evaluation of the following mating experiments were undertaken after 48 hrs of incubation. This was purposely done to allow phenotypic expression of the transferred markers. This principle was

taken into consideration especially when the transfer of antibiotic resistance was concerned. The newly introduced plasmidshad to establish themselves in the clone and at the same time produce substances or enzymes to neutralize the bactericidic action. (i. e. of the antibiotics supplemented to the agar). The inefficiency of o.n. incubation was often observed; practically no transconjugants let pin point colonies at the most could be observed.

The rate of transconjugant's formation (S. faecium 3-1pAD1tc and 25-1pAD1tc) is 5 times lower than the rate of transfer of R-Factors between members of the Enterobacteriaceae (Clowes, 1972). Our results however correlate with those of Tomich et al (1979) who transferred pAD1 (i.e. the same plasmid lacking the tetracycline transposon) with a rate of 10^{-7} per recipient CFU between two strains of S. faecalis. The latter findings exclude the reasoning that the relatively low rate of transfer is due to incompatibility betwen the two genera. (s. faecalis-S. faecium). Compared to these rates, the rates of "back" transfer of pAD1tc (and thus apparently along with the cryptic S. faecium plasmids as their mobilizing agent) to S. faecalis JH2-4 were much higher. $5 \cdot 10^{-5}$ after mating with S. faecium 25-1pAD1tc and 1:1,6 following the mating with 3-1pAD1tc.

For the purpose of selecting for JH2-4pAD1tc(25) transconjugants we searched for bacteriocin producing clones that showed the same inhibition spectrum as did S. faecium 25-1pAD1tc's bacteriocin. The derivatives this strain produced which did so were unstable. The same phenomenon was observed following the selection for transconjugants of the phenotype S. faecalis JH2-4pAD1tc(3). Novick et al (1976) refers to such phenomena as abortive transconjugants or transconjugation. The plasmids were introduced into the cell but were unable to establish in their new host as stable part of its genome. This occures among others due to repression mechanisms, replication defects caused by failiure to synthesize DNA polymerase I and III (Grinsted and Kelley, 1976), and incompatibility between plasmid and host chromosome.

Loss of genetic material in plasmids takes place in much higher frequencies than in chromosomal DNA as a consequence of them being nonessential for their bacterial host.

Plasmid loss is well known among those which are R-factors (most probably because this plasmid group has been more popular among scientists). Baron et al (1977) reported that a ß-Lactamase determining plasmid harbored by a Neisseria gonorrhoeae strain is spontaneously lost at a high rate when penicillin is omitted from the growth media. The latter observation was the cause for the addition of the appropriate antibiotics to M9 medium. This was always undertaken when plasmids with an R-Facotr character had to be isolated.

Jacob and Hobbs (1974) reported 17% loss of an antibiotic resistance plasmid (MW 50 Mdal, resistance determinants towards neomycin, streptomycin, kanamycin, tetracycline and erythromycin) harbored by S. faecalis JH2; Olsen and Shipley (1973) showed loss of R-factor DNA from Rhodospirillum and Rhodopseudomonas strains when R^+ (i.e. RP1 harboring derivatives) recipients were not under selection pressure. Gibson et al, (1979) report on the pAM 1 plasmid coding for erythromycin and lincomycin resistance. This plasmid was transferred into Lactobacillus. The group F Streptococcus strain DR 1501 pAMß1 loses the plasmid at a rate of 3-4% per generation suggesting, as the authors express themselves, that the plasmid is not stabily maintained. Here again the phenomenon is prevented by adding erythromycin and lincomycin to its growth media.

In our experiments we could not apply any selection pressure upon plasmid maintainance with bacteriocin production as marker.

In as far as loss of bacteriocinogenic plasmids is concerned - Tomich et al (1979) reported loss of pAD1 (determining ß-hemolysin and a bacteriocin) depending on the culture's age. Coetzee (1977) observed, that transductants of a Proteus mirabilis strain, which gained the ability to synthesize morganocin (plasmid determined, MW 3 Mdal), often lost their marker. Smith (1974) reported that the two plasmids coding for Col Ib and V harbored by E. coli H209 were unstabiliy maintained in this host. Col^- forms could be easily detected.

One of the factors which could have an influence upon the gap in transfer efficiencies of pAD1tc from S. faecium 25-1 and 3-1 to S. faecalis JH2-4 is the difference of grwoth rates of both strains.

Growth experiments of both strains under the same conditions showed an O.D. of 0,73 for S. faecium 3-0 and 0,54 for S. faecium 25-0 after 6 hrs. of cultivation. These observations stand in direct relation to the multiplication rate of the bacterial cells and therefore we suggest that the absolute number of S. faecium strain 25-1pAD1tc donor cells was much lower (on the filter disc) than those of S.faecium 3-1pAD1tc.

Such a high number of transconjugants following the selection for S. faecalis JH2-4pAD1tc(3) is one of the consequences of filter-mating (counter broth mating). Filter mating allows cell to cell contact to be at its optimum. Krogsted et al (1979) report of an antibiotic resistance determinant harbored by an S. faecalis strain, which has a transmission rate as high as 50%, when performing filter mating. subsequent examination of the mating mixture with the scanning electron microscope revealed intracellular connections between chains of Streptococci which were not detectable in the controls. Biswas et al (1979) observed a 1:10 rate of transfer frequency of a penicillin resistance determining plasmid harbored by a strain of Neisseria gonorrhoeae.

The introduction of pAD1tc into S. faecium 25-1 and the probable introduction of S. faecium 25-1 plasmid(s) into S. faecalis JH2-4, resulted in a broader inhibition spectrum of the bacteriocin produced by the transconjugants.

This observation suggests a synergistic interaction between each of the S. faecium bacteriocins and the pAD1tc coded one. Either the cause is of quantitative nature (more bacteriocin is produced per se) or the two bacteriocins act additively. The one gives the other access to antigene on indicator cell surface, necessary for its action. We should also mention Iyer et al (1976) in this connection. He and his research team postulate that transmissible bacterial plasmids (such as pAD1tc is) are known to bring about changes in the envelopes of their hosts. This is an aspect which should be considered a factor as well which controls efficiency of bacteriocinogenity (in all that concerns permeability).

The DNA agarose gel electrophoresis originally constitute a detection and merliminary characterization method for plasmids. It makes use of the different surface charges of plasmid DNA molecules

and separates them accoriding to their size and tertiery structural forms. The method is sensitive and does not require radioisotope labelling or density gradient centrifugation.
As will be specified below, the method however, has some limitations as well. Because of these limitations we used it only for further investigation of known plasmids. We applied this separation procedure to determine the plasmid pattern of the transconjugants (acting phenotypically as such). This was carried out after having investigated the plasmids electron microscopically. The reason stems from the fact, that OC plasmid molecules may exist in the DNA preparation. OC molecules migrate considerably more slowly than the corresponding CCC form (as determined 1972 by Aaij and Borst). Meyers et al (1976) emphasized the consequence of this phenomenon. The gel method alone does not permit a discrimination between OC and CCC DNA of previously uncharacterized isolates.
If there is doubt concerning the identity of two bands, it is possible to subject the ethanol percipitated DNA to freezing and thawing or to mild DNase treatment (Clewell and Helinski, 1969). In doing so, the CCC DNA converts to the OC form, as a result, the CCC bands disappear.
With our electrophoresis apparatus it became necessary to apply 120 volts for the first hour of the run. This was undertaken to enable plasmids with a MW greater than 25 Mdal penetrate the gel. Meyers et al (1976) noticed, that the higher the MW the more time the electrophoresis takes to make the plasmid molecules enter the gel. This time factor may be compensated for by applying a higher voltage as was done in our case. It became necessary however, to continue the run at low voltage (20 V), otherwise the plasmid DNA did not form bands but rather "half-moons" leaving a "tail" behind them.
The analysis of the transconjugants on the gel always followed a plasmid DNA enrichment procedure with DNA alkaline denaturation/ phenol extraction. This method provided us with rather stable (at -20°C) plasmid molecules, that with only one exception (the "maxi" plasmid from S. faecium 25-0) converted to the OC form.
The growth medium of the strains harboring pAD1tc was supplemented tetracycline as selection pressure and apparently a gene amplifi-

cation agent. This phenomenon was observed by Burkardet et al (1978) who cultivated E. coli harboring a tetracycline resistance determinant (plasmid pRSD1) in a medium supplemented with this antibiotic.

The migration pattern of the DNA isolated from S. faecium 3-0 and 25-0 as well as S. faecalis JH2-1 and their pAD1tc transconjugants in the electrophoresis shows the correlation between the presence of pAD1tc and the transferred phenotypical traits as determined. We have experienced that plasmids with a MW lower than 9 Mdal would band below, plasmids with higher MW in- and above the chromosomal band.As next we ececuted another mating attempt between S. faecium 3-0 and S. faecalis JH2-2. Surprisingly enough, 4,5% of the the first generation produced bacteriocin. Our previous results were reproduced hereby. It does seem that the plasmid harbored by S. faecium 3-0 codes for bacteriocin production. Furthermore, a new approach concerning non-self-transferability of "mini" plasmid is evident.

For proceeding experiments, S. faecium M16 was chosen as recipient strain. The genus homology between donor and recipient strains promised better chances for plasmid maintenance in this new host.

S. faecium M16 was characterized by Kuehnen (1980), who reports in his thesis on the presence of a 19,1 Mdal plasmid harbored by it. The strain has a high level of resistance to tetracycline and erythromycin (320 µg/ml and 256 µg/ml respectively).

It was intersting to clarify first whether or not the antibiotic reistance had anything to do with the plasmid presence. A mating with S. faecalis JH2-2 failed to produce transconjugants, however, it became evident, that genetic transfer at the chromosomal level took place: the tetracycline resistance determinants were transferred, independently of the plasmid. In curing pM16 with EB we could further prove that the erythromycin resistance determinant are located on the plasmid.

Erythromycin resistance determined by plasmids has been reported of before in this group of Streptococci. Yagi et al (1978) described a non-selftransferable 4,5 Mdal erythromycin resistance determining plasmid harbored by a S. sanguis strain. This very strain carried chromosomally located tetracycline resistance determinants.

Clewell and Franke (1974) describe a 17 Mdal plasmid harbored by a S. pyogenes strain. Gorbert and Murray (1976) report on the elimination (with EB) of a 35S plasmid harbored by S. faecalis CS-20 which carried erythromycin resistance determinants. Tomich et al (1979) report on a non-self-transferable plasmid determining the resistance against erythromycin, streptomycin and kanamycin. (MW 15 Mdal). In the same publication the authors report that the plasmid harboring strain, S. faecalis subsp. zymogenes, has a tetracycline resistance determinant located on its chromosome.
Stuy (1979) describes similar observations made with Hemophilus influenzae. The strain he worked with showed chromosomally located tetracycline and ampicillin resistance determinants. These markers were transferable to other strains of the same genus via conjugation.
As far as we can judge, no features similar to the ones previously mentioned have been shown to exist within the genus S. faecium before.

Following the phenotypic characterization of pM16 we proceeded in investigating this strain's applicability as recipient for the bacteriocin produced by S. faecium 3-0. Incidently, S. faecium M16 is susceptible to this bacteriocin so that mating mixtures containing donor:recipient in a relation of 1:10 had to be prepared. The successful cotransfer of the 3,5 Mdal S. faecium 3-0 plasmid with the capacity to synthesize bacteriocin, is a phenomenon which has not yet been described.
Transfer of "mini" plasmids without a mobilizing agent, (transfer-attempts of pM16 to S. faecium 3-1 remained fruitless), follow mechanisms the nature of which we can merely speculate on.
The problem of plasmid segregation, or rather abortive transconjugation, was already dealt with before. However, with respect to the loss of bacteriocinogenity after as many as 5 generations, one suspects that the S. faecium 3-1 plasmids have integrated into the chromosome loosing thereby their phenotypic expression. Chromosomal integration of plasmid DNA via a prophage has been observed by Schwesinger et al (1975). Upon loss of plasmid autonomy the strain reverted from thermosensitivity to thermo-stability. Yoshimoto et al (1975) described the interaction between an

R factor and its host chromosome. He and his coworkers conclude, that the R factor concerned hampers the growth of the host at high temperatures when it exists autonomously.
A similar phenomenon with closer applicability to our case is presented by Nagel de Zwaig and Puig(1964). They found that hereditary ability to synthesize bacteriocin (colicin) is transferable by cell contact among strains of the Enterobacteriaceae. Under certain conditions however (not specified), some colicinogenic factors may become fixed on the chromosome and thus cause suppression of their expressivity. Chandler (1975) reports on the integration of the host chromosome of the tetracycline resistance determining plasmid R100.1 (E. coli) and the resulting phenotypic suppresion of this resistance. Gibson (1979) applied the above in order to explain 3% loss of erythromycin resistance determining plasmid per generation.
With regards to the process of plasmid integration into the chromosome, it could be that we are confronted with a bacteriocin - transposon. Transposons or transposible genes were recognized by Cohen (1976) as components of bacterial plasmids. As far as we are informed, only transposons coding for antibiotic resistance have been reported so far. Most transposible elements contain a pair of repeated DNA regions which flank the gene and are often inverted with respect to each other. Sequences with similar properties (insertion sequences) have been observed in both bacterial chromosomes and plasmids. Starlinger (1978) and Ohtsubo and Ohtsubo (1978) brought evidence showing, that these sequences promote the formation of deletions and rearrangements of DNA regions as well as integration amd excision of bacterial plasmids.
We do not tend to relate the loss of the S. faecium 3-0 plasmid from the "temporary" transconjugants to incompatibility with the S. faecium M16 plasmid. This occures with plasmids carrying homologous nucleotide sequences (Clowes 1972) which is hardly the case in this system. The establishing of a stable transconjugant shows compatibility between both plasmids.
As to the transfer phenomenon of the mini plasmid per se, we refer to a publication of Baron et al (1977). This research team reported on somewhat similar observations which were made on a Neisseria gonorrhoeae strain, harboring three plasmids (2,7 , 4,8

and 25 Mdal in MW). The second plasmid (determining the production of ß-Lactamase) was transferred to a strain of the same genus without the 25 Mdal plasmid. The authors could not exclude, however, that this 25 Mdal plasmid interacted in this transfer since the transconjugant could never transfer the small plasmid to other recipients. Transductional transfer mechanisms could also be excluded in view of the fact that neither culture filtrates of donor cells nor chloroform treated nonviable donor cell cultures were effective in performing plasmid transfer.

Similar observations were made by Ionesno-et al (1976) and the successor research group headed by Bradford (1977). They describe a Clostridium perfringens strain harboring a 5,7 Mdal bacteriocinogenic plasmid (designated pIP404) and a 32,4 Mdal cryptic one. pIP404 was transferable per conjugation to another strain of the same genus but always alone. The authors conclude that the cryptic plasmid acted in some way as a mobilizing agent. The transduction of a R-factor into a recipient strain (Harboring pIP404 and being bacteriocinogenic) and subsequent mating with a third strain resulted in transfer of pIP404 independently of the R-factor.

As far as the literature, dealing with transfer mechanisms within the group of gram positive bacteria is concerned, very little if any information is available. The models existing today imply the theories developed for the gram negative bacteria.

Biswas et al (1979) postulate a DNA transfer via transformation, assuming that DNA is being released by cell autolysis. Gibson et al. (1979) and Jacob et al. (1975) however, could not bring the process of genetic transfer to a halt by the addition of DNase I to the mating mixture (filter method). The latter team also employed cell free filtrates of the donor strain according to the theory of DNA transfer via transformation. The test failed to confirm the theory.

In all the theories discussed above, none dealt with the role of the bacterial chromosome as transfer mediator.

Chromosomal mediated transfer of autonomous plasmid molecules (applying the existing models for the Enterobacteriaceae would mean that S. faccium 3-0 is a Hfr strain) has not yet been des-

cribed for gram positive bacteria.
Nagel de Zaig and Puig (1973) reported, that Col E1 (a plasmid with a MW of 4,2 Mdal) is transferred independently of chromosomal markers very efficiently from F^+ cells, which transfer the host chromosome in low frequencies, and from different Hfr cells. No transfer occured between F^- cells but low frequencies were observed in the transfer of Col E1 between F^- and Hfr.
Plasmid mediated chromosomal transfer in strains of S. faecalis was reported 1978 by Franke et al. The strain concerned had chromosomally located resistance markers towards streptomycin, spectinomycin and fusidic acid. Low frequencies of chromosomal marker's transfer (independently of the transfer of a mobilizing plasmid shown previously to promote transferability of non-transferable plasmids) indicated, that the markers in question have not been picked up (integrated into-) by the plasmid.
Stuy (1979) reported on a Hemophilus influenzae strain in which chromosomal resistance markers (against tetracycline and ampicillin) were transferred to a conjugative plasmid. In doing so they became transferable to other recipients of the same genus.
Hamada et al (1979) observed the transfer of histidin prototrophy to a plasmid in a strain of Agrobacterium tumefaciens.
In view of the transfer phenomenon of chromosomal segments into plasmid molecules per se, it cannot be excluded that the chromosome played a role in the transfer of the S. faecium 3-0 plasmid. The fact that no change in plasmid size was observed in the S. faecium M16/3 transconjugant could arise from incompatibility mechanisms. It should alltogether be stressed, that unfortunately none of the above mentioned publications could report on the consequences resulting from plasmid-chromosome interactions on the molecular level. This is to say that we are far from understanding the mechanisms involved in this extraordinary transfer mode of genetic material.

Plasmid determined bacteriocin production has been described before for various strains of the gram negative and gram positive bacteria. Bazaral and Helinski (1968) revealed that colicin production is determined by the plasmids Col E1 and Col E2 each with the MW of 4,2 Mdal, and Col E3 with a MW of 5 Mdal. Ionesco et

al (1976) describes a 5,7 Mdal plasmid determining bacteriocin production which is harbored by Clostridium perfringens. Lovett et al. (1977) investigated the 4,7 Mdal plasmid responsible for bacteriocin production in Bacillus pumilus. Cotzee et al (1977) described a 3 Mdal plasmid determining morganocin synthesis. A 6 Mdal plasmid which codes for the same function in Enterobacter cloacae was found by Tieze et al (1969).
As far as Streptococci of the group D are concerned, there is the publication of Jacob et al (1975) reporting on a 38 Mdal, Dunny and Clewell (1975) on a 34 Mdal and Oliver et al (1977) on a 28 Mdal plasmid determining bacteriocin production. In all three cases the strains are S. faecalis ones. In view of these works, the finding of eneterocin synthesis determining plasmid is not surprising. The plasmid size agrees well with the size of the majority of the plasmids coding for this function. It should be noted, that all the S. faecium plasmids mentioned above code also for hemolysin production - a point to be elaborated later on in this discussion.

As for the comparative studies concerning its inhibition spectrum, stability at elevated temperatures, stability towards proteolytic enzymes, pH stability as well as filterability, this enterocin fulfills the requirements proposed by Brandis and Smards (1971) for a bacteriocin. This team proposed the definition saying that bacteriocins are specific antibiotic substances produced during bacterial growth and showing a specific inhibition spectrum (effective mainly on other strains of the same genus). Furthermore, their biological active determinants have to be of protein (polypeptide) character.
The stability of the bacteriocin produced by S. faecium 3 and M16/3 at a pH as low as 1,0 is not uncommon. Weerkamp et al (1977), reported a bacteriocin (SW 31) produced by S. mutans which is stable between pH 1-10. Paul and Slade (1975) reported a bacteriocin also isolated from S. mutans, which remained stable in the range of pH 2-7. At higher pH values its titer continuely decreased until at pH 11 (with our bacteriocin it was pH 12) no activity was detectable. Tagg et al (1976) described a butyricin and perfringicin which retained activity at a pH range of 2-12, and a

boticin which retained activity with pH 1,1-9,5.

As a result of our investigations it became furthermore evident, that the bacteriocin produced by S. faecium 3-0 and S. faecium M16/3 is not identical with ß-hemolysin (as had been generally assumed of for enterococci). Brock and Davie (1963) proposed that "bacteriocin and hemolysin are probably two aspects of the same substance". Van Embden et al (1977) conclude "these observations (cotransfer of bacteriocin with hemolysin) are in agreement with the view, that the hemolysin of group D Streptococci is identical to bacteriocin". Jacob et al. (1975), Tomura et al (1973), Basinger and Jackson (1968) as well as Brefort et al. (1977) presented all the same opinion.
As a result of these mating experiments S. faecium M16/3 was shown to have the capacity to synthesize bacteriocin as well as to have an immunity against it. Bacteriocinogenity is generally known to be associated with immunity against the substance produced. The close location of the genetic determinants responsible for these functions is evidenced by their cotransfer.
Similar observations were obtained from Baquero et al. (1978) and Halwell and Sherratt (1976), who investigated the Col E1 plasmid. Jacob et al (1975) resumea that "loss of bacteriocin was accompanied by loss of immunity towards the same". Brock et al (1963), Inselburg and Johns (1975) and Sidikaro et al. (1979) discussed this phenomenon for Col E2 and Col E3. The latter team determined, that the immunity gained against Col E3 was due to a de-novo protein synthesis they were able to perform under in-vitro conditions.

Our experiments revealed, that the immunity of the S. faecium M16/3 towards the bacteriocin became exhausted when the undiluted donor strain supernatant (S. faecium 3-0) was spotted on the M16/3 lawn. Levisohn et al (1968) report on similar observations in which bacteriocin absorbed on an immune cell turned to be lethal in high concentrations. Tagg et al (1976) commence their publication with a definition of "immunity" and "resistance": bacteriocin immunity is determined as the loss of specific receptors for a particular bacteriocin. Resistance towards bacteriocins is defined as the inability of indicator cells to absorb it specifically. This research team continues with the statement, that producer cell immunity may

be incomplete.
We suggest that immunity is expressed in the ability to synthesize a protein which adheres specifically to the bacteriocin receptor structures on the surface of S. faecium M16/3 cells.
The undiluted bacteriocin of S. faecium 3-0 has probably the capacity to associate with free receptor structures and cause hereby cell death. Apparently the bacteriocin molecules are in abundance of the immunity protein synthesized by S. faecium M16/3. This is evidenced by the observation that the S. faecium 3-0 bacteriocin titer was one titer-step higher than the one from S. faecium M16/3. (S. faecium 158 employed in both cases as indicator strain).
The S. faecium M16/3 transconjugants that lost the ability to produce bacteriocin (following a maximum of 5 passages) failed to show the S. faecium 3-0 plasmid band upon electrophoretic analysis of their plasmid DNA. This fact provides further evidence for the localisation of the genetic determinants on this "mini"-plasmid.
With regard to the identity of the plasmid of donor- and recipient strains the discovery by Smith and Wilcox (1970) of restriction endonucleases has provided us with an excellent tool which helps us examine this question on the molecular level (i.e. nucleotide sequences).
We employed the Eco RI enzyme because with its activity of 2500 units it possessed the highest purity compared to all other enzymes available.
As marker for MW determination of linear DNA fragments, we employed a Hind III digested Lambda phage DNA.
The electrophoretic investigations of the cleaved plasmids confirmed the homology between the S. faecium 3-0 "mini" plasmid and the "mini" plasmid band visible in the migration track of the S. faecium M16/3 transconjugant. Depending on the amount of "back gound" DNA (i.e. chromosomal fragments) we observe a typical statistical distribution of its linear debris following the Eco RI cleavage. The migration of Lambda phage DNA debris shows a relatively high concentration of "back ground" DNA material which causes the entire track to reflect the UV light (254 nm).

The "staircase" pattern observed with the Eco RI digested plasmids is not viewed here, because purchased, pure phage DNA, probably isolated by means of a CsCl-EB-density gradient, was applied for this run. Our samples however, contained, next to plasmid DNA, chromosomal fragments as well.
Inselburg (1974) experienced, that fragments of DNA which are between 0,7 - 1,4 μ long (1,9 - 2,9 Mdal) are not clearly determineable. This is an observation we can reconfirm for the last two DNA bands representing 1,26 and 1,45 Mdal which are not as sharp as the others.
Helling et al. stated in 1974, that by increasing the voltage electrophoresis can be completed rapidly enough to allow fragments of small MW to resolve as narrow bands. However, the separation of high MW DNA becomes progressively poorer (This report correlates well with our findings). We had to remain at 22 hrs. runs at voltage as low as 20 V in order to prevent the 14,63 and 6,13 Mdal fragments to merge into one very broad band. (This causes the smaller debris to separate as sharp bands).
The slight diagonal course of the migration pattern in the slots to the extreme left and extreme right of the gel are due to the lower electrical resistance at these ends. This stems from the fact that they were the closest to the spacers, i.e. to the point, were the gel is adjacant to the buffer solution.

As we observed, the plotting of the migration distance of the different Lambda phage DNA debris against their MW is linear within a certain range only (1,5 until 6 Mdal approx.). Helling et al. (1974) as well as Thomas and Davis (1975) have performed the same electrophoresis and reported that the upper and lower limits of proportionality range (beyond the MW mentioned above) decreases progressively. This means that the relation drawn as graph (see fig. 21) would become more and more like a diagonally drawn "l".
With the aid of this reference curve we were able to determine that the S. faecium M16 plasmid was split into a 14,2 Mdal and 4,5 Mdal piece. It might be interesting to point out, that the DNA migration in gel electrophoresis depends on its GC% as postulated by Thomas and Davis (1975).
The rather surprising outcome of the conjugation experiment be-

tween S. faecium M16 and S. faecium 25-1 confronted us once again with "mini" plasmid transfer which is independent of mobilizing agents (in the sense observed until now). On the one hand we were unable to transfer genetic material from S. faecium M16 to any of the recipients employed (and therefore the characterization of the S. faecium M16 plasmid as nontransferable or noninfectuous seems justified), on the other hand this strain seems to possess outstanding recipient features. Evidently, the transfer of genetic material occured here on the plasmidlevel as will be discussed later.
The spontaneous acquisition of resistance towards rifampicin and fusidic acid occured relatively frequently. S. faecium M16 gained these features independently of the strain possessing them (when brought together in mating experiments). These observation correlate with those made by Kühnen (1980) with the same strain.

The electrophoretic investigation of the sucrose utilizing S. faecium M16/25-1 revealed that the "mini" and "midi" plasmids of the donor were cotransferred into this strain. The independent isolation of three such transconjugants, all of the same genotype, brings us to the following conclusions: 1. Neither the "mini" nor the "midi" plasmids are responsible for bacteriocin production 2. both plasmids are in some way associated with one another and are cotransferred independently of the 13 Mdal plasmid. 3. The ability to utilize sucrose is most probably located on the plasmids transferred. As for the bacteriocin production, it cannot be excluded that this feature is being coded by the "maxi" plasmid.
Plasmid cotransfer is not an unusual phenomenon (see Jorgenson et al. 1976, Tally and Snydman 1979). However, the publishers suggest that this occures as a result of a mobilizing event. This interpretation can hardly be of any relevance hereto because of the small size of the plasmids concerned.
Reviewing the interpretation of the transfer model, Guerry et al (1974) stated that nonconjugative plasmids do not have physical contact with their mobilizing agents: cotransfer seems to be independent of temporary cointegration between the two. The existing theory postulates that the transferable plasmid codes for

pili structures. The authors however, draw the conclusion that transmissibility must be reflecting something other then just a differnece in the nature of the conjugal tube. (pili).
There are some parallels between our findings and those made by Baron et al. (1977) who reported the autonomous transfer of a "mini" plasmid (2,7 Mdal) which leaves behind it a 25 Mdal cryptic plasmid in the parental cell. Eventually, the latter could be responsible for the transfer mechanisms. This cryptic plasmid we are concerned with has a MW of 13 Mdal so that the above said might be a possible interpretation of our observations.
Hamada et al (1970) Biswas et al (1979) and Franke et al (1978) reported plasmid mediated chromosomal transfer. We are convinced that those phenomena do not function in this one direction only. In postulating this, it could be argued, that S. faecium 25-0 is a Hfr strain (i.e. chromosomal mediated plasmid transfer).

The discovery of a bacterial sex hormone or the clumping inducing agent (CIA) by Dunny et al (1978) stresses the role of recipient strains in the mating process as well. It was shown that the recipient strains produce an extracellular substance that induces donor strains to become adherent in liquid media. Production of CIA is repressed in donor cells because of the presence of transferable plasmids. These findings make further investigations as to the capacity of S. faecium M16 to produce CIA necessary.

The existing homology between the S. faecium 25-1 plasmids and those presented in the plasmid migration pattern of S. faecium M16/25-1 is evident. We proceeded in restriction endonuclease digestion of the plasmids concerned. The "maxi" plasmid proves to possess two cleavage sites. The OC band this plasmid easily builds has vanished together with the CCC one to form two linear fragments.
With aid of the linear MW standards, we found them to have a relative MW of 10 and 2,6 Mdal. The two other plasmids appear unchanged in the migration track of S. faecium M16/25-1 transconjugant.

The presence of extrachromosomal DNA in bacteria and the ability to degrade substrates is not a new observation. Reports of this kind have mostly been with Pseudomonas ssp. Degradation of

salicilate was shown by Chakrabarty (1972), Campher by Rainwald et al. (1973), Naphtalene by Dunn and Gunsalus (1973), toluene and xylene by Worsey and Williams (1975) and Friello et al (1976) to stem from the presence of extrachromosomal DNA. Octane degradation was shown by Chakrabarty et al (1973) to be plasmid coded in a strain of Pseudomonas oleovorans. Fisher et al. (1978), showed that a plasmid harbored by Alkaligenes paradoxus enables this strain to utilize chlorophenoy acids. Efstathiou and Mc Kay (1976) report on plasmid determined lactose metabolism with S. lactis. Le Minor et al (1974) showed the same feature for Serratia liquefaciens to be plasmid bound. Kempler and Mc Kay (1975) reported on a 5,5 Mdal plasmid harbored by Streptococcus lactis which determines citrate utilization. Burkardt et al (1978) reported a plasmid harbored by E. coli which determines the reduction of different sulfur sources to H_2S and raffinose catabolism. The synthesis of alpha galactosidase, an invertase, and a transport system was shown to be determined by this plasmid.
The only publication on plasmid determined sucrose utilization was submitted by Le Minor et al (1973) for Salmonella. The report describes the transfer of this feature to E. coli including also a recombinant deficient strain. This, according to the authors, stress the origin of the genetic determinant for sucrose metabolism.

In attempts to support the above findings by additional experimental evidence, we proceeded in quantitative determining of this carbohydrate source during different stages of growth.
The colorimetric test was a modification of the method described by Bergmeier and Berendt (1974).
As evidenced by our measurements, the ability of S. faecium strain M16/25-1 to utilize sucrose has been (in correlation with the growth curve of this strain) more efficient than with S. faecium 25-1. The latter metabolized in 19 hrs. 7 mM compared to 10 mM utilized in 10 hrs. by the transconjugant. The relatively retarded growth of S. faecium 25-1 was experienced by us already before hand. This fastidious strain was cultivated in a relatively dull basal medium supplemented with 20 mM sucrose. Its colony size on blood agar was the smallest one of all the strains investigated in this thesis. S. faecium M16 was not tested for suc-

rose utilization capacity because it simply did not possess the ability to multiply in this medium. As examined beforehand, the Streptococci strains checked could be cultivated in peptone water only after it was supplemented with a carbohydrate source. Due to the presence of sucrose as such a source it was not surprising that the cultivation of this strain remained fruitless. In view of these findings our investigations proceeded in determining the exact traits transferred to S. faecium M16/25-1.

We chose to check the presence of ß-Fructosidase in the crude cell extract.

Cell disrupture of Streptococci is rather difficult, due among others to the round cell form. Every ml of cell suspension had to be sonified for as long as 15 min.

The enzymatic test assay was carried out in the same way as in the quantitative sucrose uptake test. Here, however, purchased ß-Fructosidase was omitted and sucrose added as substrate.

Our test system did not function in neutral pH and we had to reduce it to 4,6 in order to reach the results presented. This value is close to the one named by Barman (1969) for the same enzyme isolated from yeast and from Neurospora crasa (pH 5,0).

This is due to the fact that the reaction catalysed is a hydrolysis (Sucrose + H_2O $\xrightarrow[pH\ 4,6]{ß\text{-Fructosidase}}$ Glucose + Fructose).

Hestrin et al. (1955) recommended to perform this test at low pH. This group reports, that the activity of ß-Fructosidase from yeast shows a broad optimum range of pH 4,5-5,5, the activity tending to fall rather rapidely on the alkaline side and more slowly on the acidic side.

The colorimetric determination of reduced NADP was done only after performing 5 measurements which varied in their kinetics at a range of ± 5%. The specific activity of the enzyme (400 mU for S. faecium M16/25-1 and 390 mM of S. faecium 25-0) represents typical values for enzymes responsible for metabolic functions.

Crude cell extract isolated from S. faecium M16 failed to produce any ß-Fructosidase activity.

The characterization of this enzyme through its further purification as well as the determination of the presence of some sucrose per-

mease cotransferred will shed more light on the details of the genoytpic and phenotypic traits acquired by the transconjugant S. faecium M16/25-1

It remains unclear which one of the two plasmids code for sucrose utilization. It cannot be excluded, that both have a role in metabolizing this carbohydrate. Curing of this acquired trait or selective transformation of the plasmids (following their separation in gel electrophoresis or sucrose gradient) will certainly provide us with the appropriate answer.

SUMMARY

In the framework of this thesis we revelaed the genetic determinants located on plasmids harbored by two S. faecium strains.

Primarily we worked out a plasmid DNA enrichment procedure. This was performed with the alkaline denaturation of chromosomal DNA (pH 12,3) and deproteinization of cell material with phenol. This way plasmid DNA yeald made up 2/3 of the total DNA isolated.

The electron microscopic investigation of the plasmid DNA (employing the 13,5 μ pAD1tc plasmid as marker) revealed that the S. faecium 3-0 plasmid measured 1,7 μ , corresponding to a MW of 3,5 Mdal. The "mini", "midi" and "maxi" plasmids of S. faecium 25-0 were 1,25, 2,3 and 6,3 μ long, equivalent to 2,4, 4,7 and 13 Mdal respectively.
The determination of sedimentation coefficient of the plasmids in sucrose gradients revealed 22S for the S. faecium 3-0 plasmid, 21S for the "mini", 24S for the "midi" and 38S for the "maxi" plasmid harbored by S. faecium 25-0. These values concern the CCC plasmid form. The OC peak was demonstrated for the "maxi" plasmid and showed 29S.

The determination of their biological function was based on conjugation systems. Due to the small size of the plasmids concerned we had to first introduce a self-transferable, mobilizing plasmid into the strains harboring them. pAD1tc was employed for this purpose due to its easy selectable markers (tetracycline resistance, hemolysin production). We succeeded in transferring it into S. faecium 3-1 and S. faecium 25-1. Rate of transconjugant's formation was 10^{-7} and $1,6 \cdot 10^{-7}$ respectively. Additional evidence for transconjugant's formation was presented with the analysis of their DNA in vertical slab gel electrophoresis.
A mating experiment between these transconjugants (as donors) and the intermediating strain for the construction of isogenic pairs, S. faecalis JH2-4, followed. In both cases selection for transconjugants which gained the ability to produce bacteriocin took place. Our results pointed out that the presence of the bacteriocin determined by pAD1tc and the one produced by S. faecium 25-0 in the same bacterial cell results in a synergistic effect.

That is, inhibition spectrum and zone diameter are broadend. This phenomenon was observed for S. faecalis JH2-4pAD1tc(25) transconjugants for 2 generations. In the case of S. faecalis JH2-4pAD1tc (3) 3% of the transconjugants gained the ability to synthesize the bacteriocin produced by S. faecium 3-0 for one generation. These results were reproducible following direct mating between S. faecium 3-0 and S. faecalis JH2-2. 4,5% of the exconjugants gained the ability to produce bacteriocin, a feature they were able to retain for one generation.
Due to instability of this feature in S. faecalis, S. faecium M16 was chosen as recipient.
This strain harbors a 19,1 Mdal plasmid and is resistant to 320 µg/ml tetracycline and 256 µg/ml erythromycin. The elimination of this plasmid by employing an aged culture pretreated with ethidium bromide resulted in the loss of resistance to erythromycin. The localisation of the tetracycline resistance determinant on the chromosome was confirmed following a mating with S. faecalis JH2-2. This strain gained the resistance marker without showing plasmid bands upon electrophoretic analysis of its DNA.

In a direct mating between S. faecium 3-0 and S. faecium M16 we were able to isolate a transconjugant, capable of synthesizing the S. faecium 3-o bacteriocin. S. faecium M16/3 proved to harbor the S. faecium 3-0 "mini" plasmid following electrophoretic analysis. It could furthermore be shown, that immunity towards the bacteriocin was cotransferred and that donor- and recipient strain's bacteriocin are of the same nature (Inhibition spectra, temperature- and pH stability, filterability and susceptibility towards proteolytic enzmes). It could furthermore be demonstrated, that contrary to all other bacteriocins investigated in group D Streptococci, bacteriocinogenity is neither identical, nor associated with the production of hemolysin.
Upon cleavage of the plasmids with the Eco RI restriction endonuclease it was observed that the S. faecium 3-0 plasmid possess only one, the S. faecium M16 plasmid two sites, with the nucleotide sequence A/AGCTT (cutting it into two linear pieces of 14,2 Mdal and 4,5 Mdal).

The non-selftransmissibility of the plasmid harbored by S. faecium strain M16 as well as strain M16/3 was reconfirmed following the mating with S. faecium 3-1 and 25-1.
The latter conjugation resulted in spontaneous acquisition of resistance towards rifampicin and fusidic acid (the two antibiotic markers S. faecium 25-1 was actually selected for) and in the ability to utilize sucrose as carbohydrate source. These features correlated with the cotransfer of both the "mini" and "midi" plasmids into S. faecium M16. (as subsequently determined by electrophoretic analysis).
The search for Eco RI restriction endonuclease cleavage sites revealed that only S. faecium 25-1 "maxi" plasmid could be cut into two linear pieces (with a MW of 10 and 2,6 Mdal).

Determination of sucrose utilization per se proved, that the transconjugant, S. faecium M16/25-1, utilizes 10 mM in 9 hrs, and the donor 7 mM in 19 hrs. The recipient strain S. faecium M16 was unable to multiply in a medium containing sucrose as sole carbohydrate source.

It could furthermore be shown, that the ability to metabolize sucrose was due to the transfer of the genetic determinants for ß-Fructosidase. Our enzymatic test assay was performed using crude cell extract as enzyme source.
ß-Fructosidase determined for S. faecium 25-1 showed 390 mU/mg protein, the one determined for S. faecium M16/25-1 400 mU/mg protein.

The elimination of the ability to metabolize sucrose or the curing of S. faecium M16/25-1 from the "mini" or "midi" plamids will shed more light as to the precise localisation of the genetic determinant responsible for this trait.

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ABBREVIATIONS

CFU	-	colony forming unit
Ci	-	Curie
EB	-	ethidium bromide
PEG	-	Polyethylene glycol
o.n.	-	overnight
O.D.	-	optical density
rpm	-	revolutions per minute
Tris	-	Tris-hydroxymethyl aminomethane
EDTA	-	ethylenediamine tetraacetate
TES (buffer)	-	Tris - EDTA - sodium chloride
G-6-P-DH	-	Glucose-6-phosphate-dehydrogenase
HK	-	Hexokinase
NADP	-	Nicotinamide adeninedinucleotide phosphate
NADPH	-	reduced NADP
ATP	-	Adenosine triphosphate
MW	-	Molecular weight
CCC	-	Covalently Closed Circular
OC	-	Open Circular
TCA	-	Trichloric acetic acid
SSC	-	Saline sodium citrate
Mdal*	-	Megadalton
hrs	-	hours

* 1 Dalton is defined as a unit expressing atomic- molecular- and particular weight. It had been introduced by the british chemist and physicist John Dalton. 1 Dalton = $1.66 \cdot 10^{-27}$ kg.

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Curriculum Vitae

On the 30th of October 1949 I, Joram Keness, was born as second son to Mr. Tuvia and Mrs. Erika-Efrat Keness in Ramat-Gan, Israel. Between 1956 and 1963 I attended the elementary school in Nof-Yam. In the fall of 1963 I entered the Tichon-Chadash high-school in Tel-Aviv. In the 11th and 12th grades I majored in the natural sciences and passed in June 1967 my matriculation examinations. In August of the same year I was drafted to the israeli army. I completed my military service with the rank of staff sergeant three years later. In August 1970 I arrived in Bonn where my father was assigned to the local israeli embassy. In April 1971 I passed the preparatory college courses and began my biology studies at the Rheinische Friedrich-Wilhelms University of Bonn. In May 1973 I passed the Vordiplom examinations and in October of the same year I was granted a scholarship from the DAAD (German Academic Exchange Service). I passed the first part of my Diplom examinations in May 1975. A month later I commenced with the experiments for my Diplom thesis under the sponsorship of Prof. Dr. J. Krämer in the Institute for Medical Microbiology and Immunology. The thesis entiteled "Evidence and Characteri--zation of Plasmids in Bacteriocin Producing Strains of Streptococcus faecium" was submitted in September 1976. A month later I begun with the laboratory work which led to this thesis. During the summer of 1977 I worked as research associate for three months with the group of Prof. D.B. Clewell in the University of Michigan. In October 1976 I was granted a three-year position financed in the framework of a research grant from the DFG (German Research Council).